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(54) Title: METHYLATION SPECIFIC DETECTION

(57) Abstract

The present invention provides a method of PCR, methylation specific PCR (MSP), for rapid identification of DNA methylation patterns in a CpG-containing nucleic acid. MSP uses the PCR reaction itself to distinguish between methylated and unmethylated DNA, which adds an improved sensitivity of methylation detection.

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METHYLATION SPECIFIC DETECTION

Field of the Invention

The present invention relates generally to regulation of gene expression, and more specifically to a method of determining the DNA methylation status of CpG sites in a given locus.

Background of the Invention

In higher order eukaryotes DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide. This modification has important regulatory effects on gene expression, especially when involving CpG rich areas, known as CpG islands, located in the promoter regions of many genes. While almost all gene-associated islands are protected from methylation on autosomal chromosomes, extensive methylation of CpG islands has been associated with transcriptional inactivation of selected imprinted genes and genes on the inactive X - chromosome of females. Abberant methylation of normally unmethylated CpG islands has been described as a frequent event in immortalized and transformed cells, and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers.

Human cancer cells typically contain somatically altered genomes, characterized by mutation, amplification, or deletion of critical genes. In addition, the DNA template from human cancer cells often displays somatic changes in DNA methylation (E.R. Fearon, et al., Cell, 61:759, 1990; P.A. Jones, et al., Cancer

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Res., <u>46</u>:461, 1986; R. Holliday, *Science*, <u>238</u>:163, 1987;
A. De Bustros, *et al.*, *Proc. Natl. Acad. Sci.*, *USA*,
<u>85</u>:5693, 1988); P.A. Jones, *et al.*, *Adv. Cancer Res.*,
<u>54</u>:1, 1990; S.B. Baylin, *et al.*, *Cancer Cells*, <u>3</u>:383,
5 1991; M. Makos, *et al.*, *Proc. Natl. Acad. Sci.*, *USA*,
<u>89</u>:1929, 1992; N. Ohtani-Fujita, *et al.*, *Oncogene*,
<u>8</u>:1063, 1993). However, the precise role of abnormal DNA
methylation in human tumorigenesis has not been estab-
lished. DNA methylases transfer methyl groups from the
10 universal methyl donor S-adenosyl methionine to specific
sites on the DNA. Several biological functions have been
attributed to the methylated bases in DNA. The most
established biological function is the protection of the
DNA from digestion by cognate restriction enzymes. The
15 restriction modification phenomenon has, so far, been
observed only in bacteria. Mammalian cells, however,
possess a different methylase that exclusively methylates
cytosine residues on the DNA, that are 5' neighbors of
guanine (CpG). This methylation has been shown by
20 several lines of evidence to play a role in gene
activity, cell differentiation, tumorigenesis, X-
chromosome inactivation, genomic imprinting and other
major biological processes (Razin, A., H., and Riggs,
R.D. eds. in DNA Methylation Biochemistry and Biological
25 Significance, Springer-Verlag, New York, 1984).

A CpG rich region, or "CpG island", has recently
been identified at 17p13.3, which is aberrantly
hypermethylated in multiple common types of human cancers
(Makos, M., *et al.*, *Proc. Natl. Acad. Sci. USA*, <u>89</u>:1929,
30 1992; Makos, M., *et al.*, *Cancer Res.*, <u>53</u>:2715, 1993;
Makos, M., *et al.*, *Cancer Res.* <u>53</u>:2719, 1993). This h-
ypermethylation coincides with timing and frequency of
17p losses and p53 mutations in brain, colon, and renal

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cancers. Silenced gene transcription associated with hypermethylation of the normally unmethylated promoter region CpG islands has been implicated as an alternative mechanism to mutations of coding regions for inactivation

5 of tumor suppressor genes (Baylin, S.B., *et al.*, *Cancer Cells*, 3:383, 1991; Jones, P.A. and Buckley, J.D., *Adv. Cancer Res.*, 54:1-23, 1990). This change has now been associated with the loss of expression of VHL, a renal cancer tumor suppressor gene on 3p (J.G. Herman, *et al.*,

10 *Proc. Natl. Acad. Sci. USA*, 91:9700-9704, 1994), the estrogen receptor gene on 6q (Ottaviano, Y.L., *et al.*, *Cancer Res.*, 54:2552, 1994) and the H19 gene on 11p (Steenman, M.J.C., *et al.*, *Nature Genetics*, 7:433, 1994).

In eukaryotic cells, methylation of cytosine

15 residues that are immediately 5' to a guanosine, occurs predominantly in CG poor regions (Bird, A., *Nature*, 321:209, 1986). In contrast, discrete regions of CG dinucleotides called CpG islands remain unmethylated in normal cells, except during X-chromosome inactivation

20 (Migeon, *et al.*, *supra*) and parental specific imprinting (Li, *et al.*, *Nature*, 366:362, 1993) where methylation of 5' regulatory regions can lead to transcriptional repression. *De novo* methylation of the Rb gene has been demonstrated in a small fraction of retinoblastomas

25 (Sakai, *et al.*, *Am. J. Hum. Genet.*, 48:880, 1991), and recently, a more detailed analysis of the VHL gene showed aberrant methylation in a subset of sporadic renal cell carcinomas (Herman, *et al.*, *Proc. Natl. Acad. Sci.*, *U.S.A.*, 91:9700, 1994). Expression of a tumor suppressor

30 gene can also be abolished by *de novo* DNA methylation of a normally unmethylated 5' CpG island (Issa, *et al.*, *Nature Genet.*, 7:536, 1994; Herman, *et al.*, *supra*; Merlo, *et al.*, *Nature Med.*, 1:686, 1995; Herman, *et al.*, *Cancer*

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Res., 56:722, 1996; Graff, *et al.*, *Cancer Res.*, 55:5195, 1995; Herman, *et al.*, *Cancer Res.*, 55:4525, 1995).

Most of the methods developed to date for detection of methylated cytosine depend upon cleavage of

5   the phosphodiester bond alongside cytosine residues, using either methylation-sensitive restriction enzymes or reactive chemicals such as hydrazine which differentiate between cytosine and its 5-methyl derivative. The use of methylation-sensitive enzymes suffers from the

10   disadvantage that it is not of general applicability, since only a limited proportion of potentially methylated sites in the genome can be analyzed. Genomic sequencing protocols which identify a 5-MeC residue in genomic DNA as a site that is not cleaved by any of the Maxam Gilbert

15   sequencing reactions, are a substantial improvement on the original genomic sequencing method, but still suffer disadvantages such as the requirement for large amount of genomic DNA and the difficulty in detecting a gap in a sequencing ladder which may contain bands of varying

20   intensity.

Mapping of methylated regions in DNA has relied primarily on Southern hybridization approaches, based on the inability of methylation-sensitive restriction enzymes to cleave sequences which contain one

25   or more methylated CpG sites. This method provides an assessment of the overall methylation status of CpG islands, including some quantitative analysis, but is relatively insensitive, requires large amounts of high molecular weight DNA and can only provide information

30   about those CpG sites found within sequences recognized by methylation-sensitive restriction enzymes. A more sensitive method of detecting methylation patterns combines the use of methylation-sensitive enzymes and the

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polymerase chain reaction (PCR). After digestion of DNA
with the enzyme, PCR will amplify from primers flanking
the restriction site only if DNA cleavage was prevented
by methylation. Like Southern-based approaches, this
5   method can only monitor CpG methylation in methylation-
sensitive restriction sites. Moreover, the restriction
of unmethylated DNA must be complete, since any uncleaved
DNA will be amplified by PCR yielding a false positive
result for methylation. This approach has been useful in
10 studying samples where a high percentage of alleles of
interest are methylated, such as the study of imprinted
genes and X-chromosome inactivated genes. However,
difficulties in distinguishing between incomplete
restriction and low numbers of methylated alleles make
15 this approach unreliable for detection of tumor
suppressor gene hypermethylation in small samples where
methylated alleles represent a small fraction of the
population.
        Another method that avoids the use of restriction
20 endonucleases utilizes bisulfite treatment of DNA to
convert all unmethylated cytosines to uracil. The altered
DNA is amplified and sequenced to show the methylation
status of all CpG sites. However, this method is
technically difficult, labor intensive and without
25 cloning amplified products, it is less sensitive than
Southern analysis, requiring approximately 10% of the
alleles to be methylated for detection.
        Identification of the earliest genetic changes in
tumorigenesis is a major focus in molecular cancer
30 research. Diagnostic approaches based on identification
of these changes are likely to allow implementation of
early detection strategies and novel therapeutic

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approaches targeting these early changes might lead to
more effective cancer treatment.

Summary of the Invention

The precise mapping of DNA methylation patterns in
5   CpG islands has become essential for understanding
diverse biological processes such as the regulation of
imprinted genes, X-chromosome inactivation, and tumor
suppressor gene silencing in human cancer. The present
invention provides a method for rapid assessment of the
10   methylation status of any group of CpG sites within a CpG
island, independent of the use of methylation-sensitive
restriction enzymes. Despite the knowledge of those of
skill in the art regarding the use of PCR and the use of
bisulfite modification, independently, until the present
15   invention, no one had prepared primers that were specific
for the bisulfite reaction such that the PCR reaction
itself was used to distinguish between the chemically
modified methylated and unmethylated DNA.

The method of the invention includes modification
20   of DNA by sodium bisulfite or a comparable agent which
converts all unmethylated but not methylated cytosines to
uracil, and subsequent amplification with primers
specific for methylated versus unmethylated DNA. This
method of "methylation specific PCR" or MSP, requires
25   only small amounts of DNA, is sensitive to 0.1 % of
methylated alleles of a given CpG island locus, and can
be performed on DNA extracted from paraffin-embedded
samples, for example. MSP eliminates the false positive
results inherent to previous PCR-based approaches which
30   relied on differential restriction enzyme cleavage to
distinguish methylated from unmethylated DNA.

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   In a particular aspect of the invention, MSP is
useful for identifying promoter region hypermethylation
changes associated with transcriptional inactivation in
tumor suppressor genes, for example, p16, p15, E-cadherin
5   and VHL, in human neoplasia. Other genes that are shown
to be methylated include the estrogen receptor,MDGI, GST-
pi, calcitonin, HIC-1, endothelin B receptor, TIMP-2, 06-
MGMT, MLH1, MSH2, and GFAP.   Of those, the estrogen
receptor, MDGI, GST-pi, calcitonin, HIC-1, endothelin B
10 receptor, TIMP-2, 06-MGMT, and MLH1 were shown by MSP to
be hypermethylated in neoplastic tissue as compared with
normal tissue.   For the first time, the invention
provides evidence that TIMP-2, a tissue inhibitor of
metalloproteinases, is hypermethylated in neoplastic
15 tissue as compared with normal tissue.

Brief Description of the Drawings

   Figure 1 shows genomic sequencing of p16. The
sequence shown has the most 5' region at the bottom of
the gel, beginning at +175 in relation to a major
20 transcriptional start site (Hara, *et al., Mol. Cell
Biol.*, 16:859, 1996). All cytosines in the unmethylated
cell line H249 have been converted to thymidine, while
all C's in CpG dinucleotides in the methylated cell H157
remains as C, indicating methylation.] enclosed a *BstUI*
25 site which is at -59 in relation to the transnational
start site in Genbank sequence U12818 (Hussussian, *et
al., Nat. Genet.*, 8:15, 1994), but which is incorrectly
identified as CGCA in sequence X94154 (Hara, *et al.,
supra)*. This CGCG site represents the 3' location of the
30 sense primer used for *p16* MSP.

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Figure 2, panels A-E, show polyacrylamide gels
with the Methylation Specific PCR products of p16. Primer
sets used for amplification are designated as
unmethylated (U), methylated (M), or unmodified/wild-type
5   (W).* designates the molecular weight marker pBR322-MspI
digest. Panel A shows amplification of bisulfite-treated
DNA from cancer cell lines and normal lymphocytes, and
untreated DNA (from cell line H249). Panel B shows mixing
of various amount of H157 DNA with 1 µg of H249 DNA prior
10 to bisulfite treatment to assess the detection
sensitivity of MSP for methylated alleles. Modified DNA
from a primary lung cancer sample and normal lung are
also shown. Panel C shows amplification with the p16-U2
(U) primers, and p16-M2 (M) described in Table 1. Panel D
15 shows the amplified p16 products of panel C restricted
with BstUI(+) or not restricted (-).
Panel E shows results of testing for regional methylation
of CpG islands with MSP, using sense primers p16-U2 (U)
and p16-M2 (M), which are methylation specific, and an
20 antisense primer which is not methylation specific.

        Figure 3, panels A-E, show polyacrylamide gels of
MSP products from analysis of several genes. Primer sets
used for amplification are not designated as unmethylated
(U), methylated (M), or unmodified/wild-type (W). *
25 designates the molecular weight marker pBR322-MspI digest
and ** designates the 123bp molecular weight marker. All
DNA samples were bisulfite treated except those
designated untreated. Panel A shows the results from MSP
for p15. Panel B shows the p15 products restricted with
30 BstUI (+) or not restricted (-). Panel C shows the
products of MSP for VHL. Panel D shows the VHL products
restricted with BstUI(+) or not restricted (-). Panel E
shows the products of MSP for E-cadherin.

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Description of the Preferred Embodiments

The present invention provides methylation specific PCR (MSP) for identification of DNA methylation patterns. MSP uses the PCR reaction itself to distinguish
5   between modified methylated and unmethylated DNA, which adds an improved sensitivity of methylation detection.

Unlike previous genomic sequencing methods for methylation identification which utilizes amplification primers which are specifically designed to avoid the CpG
10 sequences, MSP primers themselves are specifically designed to recognize CpG sites to take advantage of the differences in methylation to amplify specific products to be identified by the invention assay.

As illustrated in the Examples below, MSP provides
15 significant advantages over previous PCR and other methods used for assaying methylation. MSP is markedly more sensitive than Southern analyses, facilitating detection of low numbers of methylated alleles and the study of DNA from small samples. MSP allows the study of
20 paraffin-embedded materials, which could not previously be analyzed by Southern analysis. MSP also allows examination of all CpG sites, not just those within sequences recognized by methylation-sensitive restriction enzymes. This markedly increases the number of such sites
25 which can be assessed and will allow rapid, fine mapping of methylation patterns throughout CpG rich regions. MSP also eliminates the frequent false positive results due to partial digestion of methylation-sensitive enzymes inherent in previous PCR methods for detecting
30 methylation. Furthermore, with MSP, simultaneous detection of unmethylated and methylated products in a single sample confirms the integrity of DNA as a template

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for PCR and allows a semi-quantitative assessment of allele types which correlates with results of Southern analysis. Finally, the ability to validate the amplified product by differential restriction patterns is an
5 additional advantage.

The only technique that can provide more direct analysis than MSP for most CpG sites within a defined region is genomic sequencing. However, MSP can provide similar information and has the following advantages.
10 First, MSP is much simpler and requires less than genomic sequencing, with a typical PCR and gel analysis taking 4-6 hours. In contrast, genomic sequencing, amplification, cloning, and subsequent sequencing may take days. MSP also avoids the use of expensive sequencing reagents and
15 the use of radioactivity. Both of these factors make MSP better suited for the analysis of large numbers of samples. Third, the use of PCR as the step to distinguish methylated from unmethylated DNA in MSP allows for significant increase in the sensitivity of methylation
20 detection. For example, if cloning is not used prior to genomic sequencing of the DNA, less than 10% methylated DNA in a background of unmethylated DNA cannot be seen (Myohanen, *et al., supra*). The use of PCR and cloning does allow sensitive detection of methylation patterns in
25 very small amounts of DNA by genomic sequencing (Frommer, *et al., Proc. Natl. Acad. Sci. USA*, 89:1827, 1992; Clark, *et al., Nucleic Acids Research*, 22:2990, 1994). However, this means in practice that it would require sequencing analysis of 10 clones to detect 10% methylation, 100
30 clones to detect 1% methylation, and to reach the level of sensitivity we have demonstrated with MSP (1:1000), one would have to sequence 1000 individual clones.

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In a first embodiment, the invention provides a
method for detecting a methylated CpG-containing nucleic
acid, the method including contacting a nucleic acid-
containing specimen with an agent that modifies

5 unmethylated cytosine, amplifying the CpG-containing
nucleic acid in the specimen by means of CpG-specific
oligonucleotide primers, wherein the oligonucleotide
primers distinguish between modified methylated and non-
methylated nucleic acid and detecting the methylated

10 nucleic acid.   It is understood that while the
amplification step is optional, it is desirable in the
preferred method of the invention.   The method of the
invention relies on the PCR reaction itself to
distinguish between modified (e.g., chemically modified)

15 methylated and unmethylated DNA.

The term "modifies" as used herein means the
conversion of an unmethylated cytosine to another
nucleotide which will distinguish the unmethylated from
the methylated cytosine. Preferably, the agent modifies

20 unmethylated cytosine to uracil. Preferably, the agent
used for modifying unmethylated cytosine is sodium
bisulfite, however, other agents that similarly modify
unmethylated cytosine, but not methylated cytosine can
also be used in the method of the invention.   Sodium

25 bisulfite (NaHSO$_3$) reacts readily with the 5,6-double bond
of cytosine, but poorly with methylated cytosine.
Cytosine reacts with the bisulfite ion to form a
sulfonated cytosine reaction intermediate which is
susceptible to deamination, giving rise to a sulfonated

30 uracil. The sulfonate group can be removed under alkaline
conditions, resulting in the formation of uracil. Uracil
is recognized as a thymine by Taq polymerase and
therefore upon PCR, the resultant product contains

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cytosine only at the position where 5-methylcytosine
occurs in the starting template DNA.

The primers used in the invention for
amplification of the CpG-containing nucleic acid in the
5   specimen, after bisulfite modification, specifically
distinguish between untreated or unmodified DNA,
methylated, and non-methylated DNA. MSP primers for the
non-methylated DNA preferably have a T in the 3' CG pair
to distinguish it from the C retained in methylated DNA,
10   and the compliment is designed for the antisense primer.
MSP primers usually contain relatively few Cs or Gs in
the sequence since the Cs will be absent in the sense
primer and the Gs absent in the antisense primer (C
becomes modified to U (uracil) which is amplified as T
15   (thymidine) in the amplification product).

The primers of the invention embrace o-
ligonucleotides of sufficient length and appropriate
sequence so as to provide specific initiation of
polymerization on a significant number of nucleic acids
20   in the polymorphic locus. Specifically, the term
"primer" as used herein refers to a sequence comprising
two or more deoxyribonucleotides or ribonucleotides,
preferably more than three, and most preferably more than
8, which sequence is capable of initiating synthesis of a
25   primer extension product, which is substantially
complementary to a polymorphic locus strand.
Environmental conditions conducive to synthesis include
the presence of nucleoside triphosphates and an agent for
polymerization, such as DNA polymerase, and a suitable
30   temperature and pH. The primer is preferably single
stranded for maximum efficiency in amplification, but may
be double stranded. If double stranded, the primer is
first treated to separate its strands before being used

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to prepare extension products. Preferably, the primer is
an oligodeoxy ribonucleotide. The primer must be suffi-
ciently long to prime the synthesis of extension products
in the presence of the inducing agent for polymerization.

5 The exact length of primer will depend on many factors,
including temperature, buffer, and nucleotide
composition. The oligonucleotide primer typically
contains 12-20 or more nucleotides, although it may
contain fewer nucleotides.

10   Primers of the invention are designed to be
"substantially" complementary to each strand of the
genomic locus to be amplified and include the appropriate
G or C nucleotides as discussed above. This means that
the primers must be sufficiently complementary to

15 hybridize with their respective strands under conditions
which allow the agent for polymerization to perform. In
other words, the primers should have sufficient
complementarity with the 5' and 3' flanking sequences to
hybridize therewith and permit amplification of the

20 genomic locus. While exemplary primers are provided in
SEQ ID NO:105-208, it is understood that any primer that
hybridizes with the target sequences in SEQ ID NO: 1-104
is included in the invention and is useful in the method
of the invention for detecting methylated nucleic acid,

25 as described.

        Oligonucleotide primers of the invention are
employed in the amplification process which is an
enzymatic chain reaction that produces exponential
quantities of target locus relative to the number of

30 reaction steps involved. Typically, one primer is
complementary to the negative (-) strand of the locus and
the other is complementary to the positive (+) strand.
Annealing the primers to denatured nucleic acid followed

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by extension with an enzyme, such as the large fragment
of DNA Polymerase I (Klenow) and nucleotides, results in
newly synthesized + and - strands containing the target
locus sequence. Because these newly synthesized

5   sequences are also templates, repeated cycles of
denaturing, primer annealing, and extension results in
exponential production of the region (i.e., the target
locus sequence) defined by the primer. The product of
the chain reaction is a discrete nucleic acid duplex with

10 termini corresponding to the ends of the specific primers
employed.

The oligonucleotide primers of the invention may
be prepared using any suitable method, such as
conventional phosphotriester and phosphodiester methods

15 or automated embodiments thereof. In one such automated
embodiment, diethylphosphoramidites are used as starting
materials and may be synthesized as described by
Beaucage, et al. (Tetrahedron Letters, 22:1859-1862,
1981). One method for synthesizing oligonucleotides on a

20 modified solid support is described in U.S. Patent No.
4,458,066.

Any nucleic acid specimen, in purified or
nonpurified form, can be utilized as the starting nucleic
acid or acids, provided it contains, or is suspected of

25 containing, the specific nucleic acid sequence containing
the target locus (e.g., CpG). Thus, the process may
employ, for example, DNA or RNA, including messenger RNA,
wherein DNA or RNA may be single stranded or double
stranded. In the event that RNA is to be used as a

30 template, enzymes, and/or conditions optimal for reverse
transcribing the template to DNA would be utilized. In
addition, a DNA-RNA hybrid which contains one strand of
each may be utilized. A mixture of nucleic acids may

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also be employed, or the nucleic acids produced in a
previous amplification reaction herein, using the same or
different primers may be so utilized. The specific
nucleic acid sequence to be amplified, i.e., the target

5   locus, may be a fraction of a larger molecule or can be
present initially as a discrete molecule, so that the
specific sequence constitutes the entire nucleic acid.
It is not necessary that the sequence to be amplified be
present initially in a pure form; it may be a minor

10   fraction of a complex mixture, such as contained in whole
human DNA.

     The nucleic acid-containing specimen used for
detection of methylated CpG may be from any source
including brain, colon, urogenital, hematopoietic,

15   thymus, testis, ovarian, uterine, prostate, breast,
colon, lung and renal tissue and may be extracted by a
variety of techniques such as that described by Maniatis,.
et al. (Molecular Cloning: A Laboratory Manual, Cold
Spring Harbor, NY, pp 280, 281, 1982).

20   If the extracted sample is impure (e.g., plasma,
serum, stool, ejaculate, sputum, saliva, cerebrospinal
fluid or blood or a sample embedded in parrafin), it may
be treated before amplification with an amount of a
reagent effective to open the cells, fluids, tissues, or

25   animal cell membranes of the sample, and to expose and/or
separate the strand(s) of the nucleic acid(s). This
lysing and nucleic acid denaturing step to expose and
separate the strands will allow amplification to occur
much more readily.

30   Where the target nucleic acid sequence of the
sample contains two strands, it is necessary to separate
the strands of the nucleic acid before it can be used as
the template. Strand separation can be effected either

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as a separate step or simultaneously with the synthesis
of the primer extension products. This strand separation
can be accomplished using various suitable denaturing
conditions, including physical, chemical, or enzymatic

5 means, the word "denaturing" includes all such means.
One physical method of separating nucleic acid strands
involves heating the nucleic acid until it is denatured.
Typical heat denaturation may involve temperatures
ranging from about 80° to 105°C for times ranging from

10 about 1 to 10 minutes. Strand separation may also be
induced by an enzyme from the class of enzymes known as
helicases or by the enzyme RecA, which has helicase
activity, and in the presence of riboATP, is known to
denature DNA. The reaction conditions suitable for

15 strand separation of nucleic acids with helicases are
described by Kuhn Hoffmann-Berling (CSH-Quantitative
Biology, 43:63, 1978) and techniques for using RecA are
reviewed in C. Radding (*Ann. Rev. Genetics*, 16:405-437,
1982).

20   When complementary strands of nucleic acid or
acids are separated, regardless of whether the nucleic
acid was originally double or single stranded, the
separated strands are ready to be used as a template for
the synthesis of additional nucleic acid strands. This

25 synthesis is performed under conditions allowing
hybridization of primers to templates to occur.
Generally synthesis occurs in a buffered aqueous
solution, preferably at a pH of 7-9, most preferably
about 8. Preferably, a molar excess (for genomic nucleic

30 acid, usually about 10^8:1 primer:template) of the two
oligonucleotide primers is added to the buffer containing
the separated template strands. It is understood,
however, that the amount of complementary strand may not

be known if the process of the invention is used for
diagnostic applications, so that the amount of primer
relative to the amount of complementary strand cannot be
determined with certainty.   As a practical matter,

5 however, the amount of primer added will generally be in
molar excess over the amount of complementary strand
(template) when the sequence to be amplified is contained
in a mixture of complicated long-chain nucleic acid
strands.   A large molar excess is preferred to improve

10 the efficiency of the process.

The deoxyribonucleoside triphosphates dATP, dCTP,
dGTP, and dTTP are added to the synthesis mixture, either
separately or together with the primers, in adequate
amounts and the resulting solution is heated to about

15 90°-100°C from about 1 to 10 minutes, preferably from 1 to
4 minutes.   After this heating period, the solution is
allowed to cool to room temperature, which is preferable
for the primer hybridization.   To the cooled mixture is
added an appropriate agent for effecting the primer

20 extension reaction (called herein "agent for
polymerization"), and the reaction is allowed to occur
under conditions known in the art.   The agent for
polymerization may also be added together with the other
reagents if it is heat stable.   This synthesis (or

25 amplification) reaction may occur at room temperature up
to a temperature above which the agent for polymerization
no longer functions.   Thus, for example, if DNA
polymerase is used as the agent, the temperature is
generally no greater than about 40°C.   Most conveniently

30 the reaction occurs at room temperature.

The agent for polymerization may be any compound
or system which will function to accomplish the synthesis
of primer extension products, including enzymes.

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Suitable enzymes for this purpose include, for example,
E. coli DNA polymerase I, Klenow fragment of *E. coli* DNA
polymerase I, T4 DNA polymerase, other available DNA
polymerases, polymerase muteins, reverse transcriptase,

5 and other enzymes, including heat-stable enzymes (*i.e.*,
those enzymes which perform primer extension after being
subjected to temperatures sufficiently elevated to cause
denaturation). Suitable enzymes will facilitate
combination of the nucleotides in the proper manner to

10 form the primer extension products which are
complementary to each locus nucleic acid strand.
Generally, the synthesis will be initiated at the 3' end
of each primer and proceed in the 5' direction along the
template strand, until synthesis terminates, producing

15 molecules of different lengths. There may be agents for
polymerization, however, which initiate synthesis at the
5' end and proceed in the other direction, using the same
process as described above.

         In nucleic acid hybridization reactions, the
20 conditions used to achieve a particular level of
stringency will vary, depending on the nature of the
nucleic acids being hybridized. For example, the length,
degree of complementarity, nucleotide sequence
composition (*e.g.*, GC v. AT content), and nucleic acid

25 type (*e.g.*, RNA v. DNA) of the hybridizing regions of the
nucleic acids can be considered in selecting
hybridization conditions. An additional consideration is
whether one of the nucleic acids is immobilized, for
example, on a filter.

30   An example of progressively higher stringency
conditions is as follows: 2 x SSC/0.1% SDS at about room
temperature (hybridization conditions); 0.2 x SSC/0.1%
SDS at about room temperature (low stringency

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conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate
stringency conditions); and 0.1 x SSC at about 68°C (high
stringency conditions). Washing can be carried out using
only one of these conditions, e.g., high stringency

5 conditions, or each of the conditions can be used, e.g.,
for 10-15 minutes each, in the order listed above,
repeating any or all of the steps listed. However, as
mentioned above, optimal conditions will vary, depending
on the particular hybridization reaction involved, and

10 can be determined empirically.

Preferably, the method of amplifying is by PCR, as
described herein and as is commonly used by those of
ordinary skill in the art. Alternative methods of
amplification have been described and can also be

15 employed as long as the methylated and non-methylated
loci amplified by PCR using the primers of the invention
is similarly amplified by the alternative means.

The amplified products are preferably identified
as methylated or non-methylated by sequencing. Sequences

20 amplified by the methods of the invention can be further
evaluated, detected, cloned, sequenced, and the like,
either in solution or after binding to a solid support,
by any method usually applied to the detection of a
specific DNA sequence such as PCR, oligomer restriction

25 (Saiki, et al., Bio/Technology, 3:1008-1012, 1985),
allele-specific oligonucleotide (ASO) probe analysis
(Conner, et al., Proc. Natl. Acad. Sci. USA, 80:278,
1983), oligonucleotide ligation assays (OLAs) (Landegren,
et al., Science, 241:1077, 1988), and the like.

30 Molecular techniques for DNA analysis have been reviewed
(Landegren, et al., Science, 242:229-237, 1988).

Optionally, the methylation pattern of the nucleic
acid can be confirmed by restriction enzyme digestion and

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Southern blot analysis. Examples of methylation
sensitive restriction endonucleases which can be used to
detect 5'CpG methylation include *SmaI*, *SacII*, *EagI*,
MspI, *HpaII*, *BstUI* and *BssHII*, for example.

5   The invention provides a method for detecting a
cell having a methylated CpG island or a cell
proliferative disorder associated with methylated CpG in
a tissue or biological fluid of a subject, comprising
contacting a target cellular component suspected of
10 expressing a gene having a methylated CpG or having a
CpG-associated disorder, with an agent which binds to the
component. The target cell component can be nucleic
acid, such as DNA or RNA, or protein. When the component
is nucleic acid, the reagent is a nucleic acid probe or
15 PCR primer. When the cell component is protein, the
reagent is an antibody probe. The probes can be
detectably labeled, for example, with a radioisotope, a
fluorescent compound, a bioluminescent compound, a
chemiluminescent compound, a metal chelator, or an
20 enzyme. Those of ordinary skill in the art will know of
other suitable labels for binding to the antibody, or
will be able to ascertain such, using routine
experimentation.

          Actively transcribed genes generally contain fewer
25 methylated CGs than the average number in DNA.
Hypermethylation can be detected by restriction
endonuclease treatment and Southern blot analysis.
Therefore, in a method of the invention, when the
cellular component detected is DNA, restriction
30 endonuclease analysis is preferable to detect
hypermethylation of the promoter for example. Any
restriction endonuclease that includes CG as part of its
recognition site and that is inhibited when the C is

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methylated, can be utilized. Preferably, the methylation
sensitive restriction endonuclease is *BssH*II, *Msp*I, or
*Hpa*II, used alone or in combination. Other methylation
sensitive restriction endonucleases will be known to
5   those of skill in the art.

          For purposes of the invention, an antibody or
nucleic acid probe specific for a gene or gene product
may be used to detect the presence of methylation either
by detecting the level of polypeptide (using antibody) or
10 methylation of the polynucleotide (using nucleic acid
probe) in biological fluids or tissues. For antibody
based detection, the level of the polypeptide is compared
with the level of polypeptide found in a corresponding
"normal" tissue. Oligonucleotide primers based on any
15 coding sequence region of the promoter in the TIMP-2,
estrogen receptor, GST-pi, calcitonin, HIC-1 or MLH1
sequence, for example, are useful for amplifying DNA, for
example by PCR. These genes are merely listed as
examples and are not meant to be limiting. Any specimen
20 containing a detectable amount of polynucleotide or
antigen can be used.   Preferably the subject is human.

          The present invention provides the finding that
TIMP-2 is methylated in cancer tissue as compared to
normal tissue. For example, TIMP-2 was found to be
25 methylated in colon cancer tissue but not in normal colon
tissue. The method for detecting a cell expressing a
gene such as TIMP-2, or a cell proliferative disorder
associated with methylation of CpG containing TIMP-2, or
any gene including those described above, can be utilized
30 for detection of residual cancer or other malignancies in
a subject in a state of clinical remission.
Additionally, the method for detecting polypeptide in
cells is useful for detecting a cell proliferative

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disorder by measuring the level of polypeptide in cells
expressing the polypeptide, in a suspect tissue in
comparison with the polypeptide expressed in normal cells
or tissue. Using the method of the invention, expression
5   of any gene, such as TIMP-2, can be identified in a cell
and the appropriate course of treatment can be employed
(e.g., sense gene therapy or drug therapy). The
expression pattern of the gene, e.g., TIMP-2, may vary
with the stage of malignancy of a cell, therefore, a
10   sample such as breast or colon tissue can be screened
with a panel of gene or gene product specific reagents
(i.e., nucleic acid probes or antibodies) to detect gene
expression, e.g., TIMP-2, and diagnose the stage of
malignancy of the cell.

15   Monoclonal antibodies can be used in the method of
the invention, for example, in immunoassays in liquid
phase or bound to a solid phase carrier. In addition,
the monoclonal antibodies in these immunoassays can be
detectably labeled in various ways. Examples of types of
20   immunoassays which can utilize monoclonal antibodies of
the invention are competitive and non-competitive
immunoassays in either a direct or indirect format.
Examples of such immunoassays are the radioimmunoassay
(RIA) and the sandwich (immunometric) assay. Detection
25   of the antigens using the monoclonal antibodies of the
invention can be done utilizing immunoassays which are
run in either the forward, reverse, or simultaneous
modes, including immunohistochemical assays on
physiological samples. Those of skill in the art will
30   know, or can readily discern, other immunoassay formats
without undue experimentation.

          The term "immunometric assay" or "sandwich
immunoassay", includes simultaneous sandwich, forward

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sandwich and reverse sandwich immunoassays. These terms
are well understood by those skilled in the art. Those
of skill will also appreciate that antibodies according
to the present invention will be useful in other
5 variations and forms of assays which are presently known
or which may be developed in the future. These are
intended to be included within the scope of the present
invention.

     Monoclonal antibodies can be bound to many
10 different carriers and used to detect the presence of
TIMP-2. Examples of well-known carriers include glass,
polystyrene, polypropylene, polyethylene, dextran, nylon,
amylases, natural and modified celluloses,
polyacrylamides, agaroses and magnetite. The nature of
15 the carrier can be either soluble or insoluble for
purposes of the invention. Those skilled in the art will
know of other suitable carriers for binding monoclonal
antibodies, or will be able to ascertain such using
routine experimentation.

20   For purposes of the invention, TIMP-2 may be
detected by the monoclonal antibodies when present in
biological fluids and tissues. Any sample containing a
detectable amount of TIMP-2 can be used. A sample can be
a liquid such as ejaculate, urine, saliva, cerebrospinal
25 fluid, blood, serum and the like, or a solid or semi-
solid such as tissues, feces, and the like, or,
alternatively, a solid tissue such as those commonly used
in histological diagnosis.

     In performing the assays it may be desirable to
30 include certain "blockers" in the incubation medium
(usually added with the labeled soluble antibody). The
"blockers" are added to assure that non-specific
proteins, proteases, or anti-heterophilic immunoglobulins

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to anti-TIMP-2 immunoglobulins present in the
experimental sample do not cross-link or destroy the
antibodies on the solid phase support, or the
radiolabeled indicator antibody, to yield false positive

5　or false negative results. The selection of "blockers"
therefore may add substantially to the specificity of the
assays described in the present invention.

In using a monoclonal antibody for the *in vivo*
detection of antigen, the detectably labeled monoclona l

10　antibody is given in a dose which is diagnostically
effective. The term "diagnostically effective" means
that the amount of detectably labeled monoclonal antibody
is administered in sufficient quantity to enable
detection of the site having the TIMP-2 antigen for which

15　the monoclonal antibodies are specific.

The concentration of detectably labeled monoclonal
antibody which is administered should be sufficient such
that the binding to those cells having TIMP-2 is
detectable compared to the background. Further, it is

20　desirable that the detectably labeled monoclonal antibody
be rapidly cleared from the circulatory system in order
to give the best target-to-background signal ratio.

As a rule, the dosage of detectably labeled
monoclonal antibody for *in vivo* diagnosis will vary

25　depending on such factors as age, sex, and extent of
disease of the individual. The dosage of monoclonal
antibody can vary from about 0.001 mg/m^2 to about 500
mg/m^2, preferably 0.1 mg/m^2 to about 200 mg/m^2, most
preferably about 0.1 mg/m^2 to about 10 mg/m^2. Such

30　dosages may vary, for example, depending on whether
multiple injections are given, tumor burden, and other
factors known to those of skill in the art.

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For *in vivo* diagnostic imaging, the type of
detection instrument available is a major factor in
selecting a given radioisotope. The radioisotope chosen
must have a type of decay which is detectable for a given
5   type of instrument. Still another important factor in
selecting a radioisotope for *in vivo* diagnosis is that
the half-life of the radioisotope be long enough so that
it is still detectable at the time of maximum uptake by
the target, but short enough so that deleterious
10   radiation with respect to the host is minimized.
Ideally, a radioisotope used for *in vivo* imaging will
lack a particle emission, but produce a large number of
photons in the 140-250 keV range, which may be readily
detected by conventional gamma cameras.

15   A monoclonal antibody useful in the method of the
invention can also be labeled with a paramagnetic isotope
for purposes of *in vivo* diagnosis, as in magnetic
resonance imaging (MRI) or electron spin resonance (ESR).
In general, any conventional method for visualizing
20   diagnostic imaging can be utilized. Usually gamma and
positron emitting radioisotopes are used for camera
imaging and paramagnetic isotopes for MRI. Elements
which are particularly useful in such techniques include
^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

25   Monoclonal antibodies used in the method of the
invention can be used to monitor the course of
amelioration of TIMP-2 associated cell proliferative
disorder. Thus, by measuring the increase or decrease in
the number of cells expressing TIMP-2 or changes in TIMP-
30   2 present in various body fluids, it would be possible to
determine whether a particular therapeutic regiment aimed
at ameliorating the disorder is effective.

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The term "modulate" envisions the suppression of methylation of TIMP-2 (e.g., promoter) or augmentation of TIMP-2 gene expression when TIMP-2 is under-expressed. When a cell proliferative disorder is associated with

5 TIMP-2 expression, such methylation suppressive reagents as 5-azacytadine can be introduced to a cell. Alternatively, when a cell proliferative disorder is associated with under-expression of TIMP-2 polypeptide, a sense polynucleotide sequence (the DNA coding strand)

10 encoding the promoter region or the promoter operably linked to the structural gene, or TIMP-2 polypeptide can be introduced into the cell.

The present invention also provides gene therapy for the treatment of cell proliferative disorders which

15 are mediated by TIMP-2. Such therapy would achieve its therapeutic effect by introduction of the appropriate TIMP-2 polynucleotide which contains either a normal TIMP-2 promoter region alone or in combination with a TIMP-2 structural gene (sense), into cells of subjects

20 having the proliferative disorder. Alternatively, the TIMP-2 structural gene could be introduced operably linked to a heterologous promoter. Delivery of sense TIMP-2 promoter polynucleotide constructs can be achieved using a recombinant expression vector such as a chimeric

25 virus or a colloidal dispersion system.

The promoter polynucleotide sequences used in the method of the invention may be the native, unmethylated sequence or, alternatively, may be a sequence in which a nonmethylatable analog is substituted within the

30 sequence. Preferably, the analog is a nonmethylatable analog of cytidine, such as 5-azacytadine. Other analogs will be known to those of skill in the art. Alternatively, such nonmethylatable analogs could be

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administered to a subject as drug therapy, alone or
simultaneously with a sense promoter for TIMP-2 or a
sense promoter operably linked with the structural gene
for the corresponding gene (e.g., TIMP-2 promoter with
5 TIMP-2 structural gene).

The invention also relates to a medicament or
pharmaceutical composition comprising a TIMP-2 promoter
polynucleotide or a TIMP-2 promoter polynucleotide
operably linked to the TIMP-2 structural gene,
10 respectively, in a pharmaceutically acceptable excipient
or medium wherein the medicament is used for therapy of
TIMP-2 associated cell proliferative disorders.

The invention also provides the use of MSP for in
situ methylation analysis. For example, MSP can be used
15 to detect methylation of DNA in the nucleus of an intact
cell. A tissue section, a cell or population of cells is
placed or immobilized on a solid support (e.g., a slide)
and MSP primers used directly on the cell for
amplification of the appropriate sequences. The primers
20 are typically detectably labeled with a reporter means,
e.g., fluorescent label. Alternatively, a probe that
detects or hybridizes with the MSP amplified sequences is
used to detect amplification of methylated sequences. In
situ methylation analysis using MSP is useful, for
25 example, in detecting nucleic acid having a mutant
nucleotide sequence associated with a primary tumor in
the adjacent histopathologic surgical margins and more
distant tissues, such as regional lymph nodes, which are
apparently "normal" when examined by standard
30 histological techniques. Using MSP, it is possible to
detect target nucleic acids from cells previously
associated with a large number of disease states which
are present in tissue that appears normal. MSP in situ

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can be used as an adjunct to cytopathology, to screen
high-risk populations and to monitor high risk patients
undergoing chemoprevention or chemotherapy.

    Exemplary target polynucleotide sequences to which
5 the primer of the invention hybridizes have a sequence as
listed below.

		SEQ ID NO.
Wild type p16	5'-GCGGTCCGCCCCACCCTCTG-3';	1
10	5'-CCACGGCCGCGGCCCG- 3';	2
Methylated p16-1*	5'-GCGATCCGCCCCACCCTCTAATAA-3';	3
	5'-TTACGGTCGCGGTTCGGGGTC-3';	4
Unmethylated p16-1	5'-ACAATCCACCCCACCCTCTAATAA-3';	5
	5'-TTATGGTTGTGGTTTGGGGTTG-3';	6
15 Methylated p16-2	5'-GCGATCCGCCCCACCCTCTAATAA-3';	7
	5'-CGGTCGGAGGTCGATTTAGGTGG-3';	8
Unmethylated p16-2	5'-ACAATCCACCCCACCCTCTAATAA-3';	9
	5'-TGGTTGGAGGTTGATTTAGGTGG-3';	10
Wild type p15	5'-TCTGGCCGCAGGGTGCG-3';	11
20	5'-CCGGCCGCTCGGCCACT-3';	12
Methylated p15	5'-AACCGCAAAATACGAACGC-3';	13
	5'-TCGGTCGTTCGGTTATTGTACG-3';	14
Unmethylated p15	5'-AACCACAAAATACAAACACATCACA-3;	15
	5'-TTGGTTGTTTGGTTATTGTATGG-3';	16
25 Methylated VHL	5'-GCGTACGCAAAAAAATCCTCCA-3';	17
	5'-TTCGCGGCGTTCGGTTC-3';	18
Unmethylated VHL	5'-ACATACACAAAAAAATCCTCCAAC-3';	19
	5'-TTTGTGGTGTTTGGTTTGGG-3';	20

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| Methylated E-cadherin | 5'-ACGCGATAACCCTCTAACCTAA-3'; | 21 |
| | 5'-GTCGGTAGGTGAATTTTTAGTTA-3'; | 22 |

| Unmethylated E-cadherin | 5'-ACAATAACCCTCTAACCTAAAATTA-3'; | 23 |
| | 5'-TGTGTTGTTGATTGGTTGTG-3'; | 24 |

| Methylated Androgen Receptor | 5'-GCGACCTCTAAATACCTAAAACCC-3'; | 25 |
| | 5'-CGTAGAGGTTTTATAGGTTATTTGGA-3'; | 26 |

| Unmethylated Androgen Receptor | 5'-ACAACCTCTAAATACCTAAAACCC-3'; | 27 |
| | 5'-TGTAGAGGTTTTATAGGTTATTTGGT-3'; | 28 |

| Methylated Estrogen Receptor | 5'-GACGAACTTACTACTATCCAAATACAC-3'; | 29 |
| | 5'-TTTACGGTTAGATCGGTTTTTTTTACG-3'; | 30 |

| Unmethylated Estrogen Receptor | 5'-AACAAACTTACTACTATCCAAATACACC-3'; | 31 |
| | 5'-TGGTTAGATTGGTTTTTTTTATGG-3'; | 32 |

| Methylated MDGI | 5'-GCCCCCGACTCCCGAAATAAA-3'; | 33 |
| | 5'-CGTCGTCGGAGTTTTTGTACGTT-3'; | 34 |

| Unmethylated MDGI | 5'-ACCCCCAACTCCCAAAATAAAAAA-3'; | 35 |
| | 5'-TGTTGTTGGAGTTTTTGTATGTTT-3'; | 36 |

| Methylated GSTp | 5'-GACGACCGCTACACCCCGAA-3'; | 37 |
| | 5'-CGTCGTGATTTTAGTATTGGGGC-3'; | 38 |

| Unmethylated GSTp | 5'-AACAACCACTACACCCCAAACATC-3'; | 39 |
| | 5'-TGTTGTGATTTTAGTATTGGGGTGG-3'; | 40 |

| Methylated Calcitonin | 5'-GCCAACGACTACTCTTATTCCCG-3'; | 41 |
| | 5'-CGTCGTCGTTTTTATAGGGTTTTG-3'; | 42 |

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Unmethylated Calcitonin	5'-ACCAACAACTACTCTTATTCCCACC-3';	43
	5'-TGTTGTTGTTTTTATAGGGTTTTGG-3';	44
Methylated HIC-1	5'-GACGCACAACCGACTACGAC-3';	45
	5'-CGGCGTTAGGGCGGGTATC-3';	46
Unmethylated HIC-1	5'-AACACACAACCAACTACAACCC-3';	47
	5'-TGGTGTTAGGGTGGGTATTGTG-3';	48
Methylated Endothelin	5'-GCGTAACCAAAAAAAATAAATAATATAC-3';	49
	5'-CGCGTTGGTGAGTTATGA-3';	50
Unmethylated Endothelin	5'-ACATAACCAAAAAAAATAAATAATATACAA-3';	51
	5'-TGTGTTGGTGAGTTATGAGTGTTAAG-3';	52
Methylated TIMP-2	5'-GACCGCGCTACCTTCTACGAATAT-3';	53
	5'-CGCGGGAGGGGTTCGTT-3';	54
Unmethylated TIMP-2	5'-AACCACACTACCTTCTACAAATATATTTACTA-3';	55
	5'-TGTGGGAGGGGTTTGTTTTG-3';	56
Methylated MLH1-a	5'-GCGACCCTAATAAAACGTCTACGT-3';	57
	5'-CGCGGGTAGTTACGATGAGG-3';	58
Unmethylated MLH1-a	5'-ACAACCCTAATAAAACATCTACATCAAAA-3';	59
	5'-TGTGGGTAGTTATGATGAGGTGGT-3';	60
Methylated MLH1-b	5'-GAACGACATTTTAACGCCAAAAA-3';	61
	5'-CGGCGGGGGAAGTTATTTA-3';	62
Unmethylated MLH1-b	5'-AAACAACATTTTAACACCAAAAAAACC-3';	63
	5'-TGGTGGGGGAAGTTATTTAGTGG-3';	64
Methylated MSH2	5'-GAACGACGTCCGACCACGA-3';	65
	5'-CGGTGTAGTCGAAGGAGACGTTG-3';	66
Unmethylated MSH2	5'-AAACAACATCCAACCACAACAACC-3';	67
	5'-TGGTGTAGTTGAAGGAGATGTTGTAGTTG-3';	68

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Methylated GFAP	5'-GATACCCGAATACCCCTAACAAC-3';	69
	5'-CGTCGTTTTTACGTTTTTTTAGGG-3';	70
Unmethylated GFAP	5'-AATACCCAAATACCCCTAACAACA-3'	71
	5'-TGTTGTTTTTATGTTTTTTTAGGGGA-3'	72
Methylated TGFb1	5'-GCGAACTACCAAACGAACCCA-3';	73
	5'-CGCGGCGGTTAGGGAGG-3';	74
Unmethylated TGFb1	5'-ACAAACTACCAAACAAACCCAACC-3';	75
	5'-TGTGGTGGTTAGGGAGGTGGG-3';	76
Methylated TGFb2	5'-GCGCGAAAATATCGTCG-3';	77
	5'-CGCGTTTCGTCGGTTT-3';	78
Unmethylated TGFb2	5'-ACACAAAAATATCATCACTCCATAC-3';	79
	5'-TGTGTTTTGTTGGTTTTTAGGT-3';	80
Methylated p130	5'-GACGCTAACCGCCTACAAACA-3';	81
	5'-CGGTCGTTTAGGGGTGCGT-3';	82
Unmethylated p130	5'-AACACTAACCACCTACAAACACCCA-3';	83
	5'-TGGTTGTTTAGGGGTGTGTTATGTT-3';	84
Methylated BRCA2	5'-GACTCCGCCTCTACCGC-3';	85
	5'-CGGTTTTTGTTAGTTTATTTCG-3';	86
Unmethylated BRCA2	5'-AACTCCACCTCTACCACCTAAT-3';	87
	5'-TGGTTTTTGTTAGTTTATTTTGG-3';	88
Methylated O6-MGMT	5'-GCGCGAAAACGAAACCGA-3';	89
	5'-CGCGTTTCGGATATGTTGGG-3';	90
Unmethylated O6-MGMT	5'-ACACAAAAACAAAACCAAAACAC-3';	91
	5'-TGTGTTTTGGATATGTTGGGA-3';	92
Methylated NF1	5'-GAACGTCCCTCAACGCCGTAA-3';	93
	5'-CGTATGCGCGGTAGGTCGTTT-3';	94

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Unmethylated NF1	5'-AAACATCCCTCAACACCATAAAACTC-3';	95
	5'-TGTATGTGTGGTAGGTTGTTTTTTTTTTT-3';	96
Methylated NF2	5'-GCGAAACTCAAACCCGAAAC-3';	97
	5'-CGTTTATCGTGAGGATCGTTATTAT-3';	98
Unmethylated NF2	5'-ACAAAACTCAAACCCAAAACCC-3';	99
	5'-TGTTTATTGTGAGGATTGTTATTATGG-3';	100
Methylated TSG101	5'-GCTACTAAACTACCCCAAACCGTC-3';	101
	5'-CGGTCGTTATGGCGGTGTC-3';	102
Unmethylated TSG101	5'-ACTACTAAACTACCCCAAACCATCC-3';	103
	5'-TGGTTGTTATGGTGGTGTTGGAG-3';	104

Exemplary primer pairs included in the invention
that hybridize to the above sequences include:

	SEQ ID NO:
5'-CAGAGGGTGGGGCGGACCGC-3' and	105
5'-CGGGCCGCGGCCGTGG-3';	106
5'-TTATTAGAGGGTGGGGCGGATCGC-3' and	107
5'-GACCCCGAACCGCGACCGTAA-3';	108
5'-TTATTAGAGGGTGGGGTGGATTGT-3' and	109
5'-CAACCCCAAACCACAACCATAA-3';	110
5'-TTATTAGAGGGTGGGGCGGATCGC-3' and	111
5-CCACCTAAATCGACCTCCGACCG-3';	112
5'-TTATTAGAGGGTGGGGTGGATTGT-3' and	113
5'-CCACCTAAATCAACCTCCAACCA-3';	114
5'-CGCACCCTGCGGCCAGA-3' and	115
5'-AGTGGCCGAGCGGCCGG-3';	116
5'-GCGTTCGTATTTTGCGGTT-3' and	117
5'-CGTACAATAACCGAACGACCGA-3';	118

30

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5'-TGTGATGTGTTTGTATTTTGTGGTT-3' and   119
5'-CCATACAATAACCAAACAACCAA-3';   120

5'-TGGAGGATTTTTTTGCGTACGC-3' and   121
5'-GAACCGAACGCCGCGAA-3';   122

5   5'-GTTGGAGGATTTTTTTGTGTATGT-3' and   123
5'-CCCAAACCAAACACCACAAA-3';   124

5'-TTAGGTTAGAGGGTTATCGCGT-3' and   125
5'-TAACTAAAAATTCACCTACCGAC-3';   126

5'-TAATTTTAGGTTAGAGGGTTATTGT-3' and   127
10   5'-CACAACCAATCAACAACACA-3'   128

5'GGGTTTTAGGTATTTAGAGGTCGC-3' and   129
5'ACCAAATAACCTATAAAACCTCTACG-3'   130

5'GGGTTTTAGGTATTTAGAGGTTGT-3' and   131
15   5'ACCAAATAACCTATAAAACCTCTACA-3'   132

5'GTGTATTTGGATAGTAGTAAGTTCGTC-3' and   133
5'CGTAAAAAAAACCGATCTAACCGTAAA-3'   134

5'GGTGTATTTGGATAGTAGTAAGTTTGTT-3' and   135
20   5'CCATAAAAAAAACCAATCTAACCA-3'   136

5'TTTATTTCGGGAGTCGGGGGC-3' and   137
5'AACGTACAAAAACTCCGACGACG-3'   138

5'TTTTTTATTTTGGGAGTTGGGGGT-3' and   139
25   5'AAACATACAAAAACTCCAACAACA-3'   140

5'TTCGGGGTGTAGCGGTCGTC-3' and   141
5'GCCCCAATACTAAAATCACGACG-3'   142

5'GATGTTTGGGGTGTAGTGGTTGTT-3' and   143
30   5'CCACCCCAATACTAAAATCACAACA-3'   144

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5' CGGGAATAAGAGTAGTCGTTGGC-3' and   145
5' CAAAACCCTATAAAAACGACGACG-3'   146

5' GGTGGGAATAAGAGTAGTTGTTGGT-3' and   147
5' CCAAAACCCTATAAAAACAACAACA-3'   148

5' GTCGTAGTCGGTTGTGCGTC-3' and   149
5' GATACCCGCCCTAACGCCG-3'   150

5' GGGTTGTAGTTGGTTGTGTGTT-3' and   151
5' CACAATACCCACCCTAACACCA-3'   152

5' GTATATTATTTATTTTTTTTGGTTACGC-3' and   153
5' TCATAACTCGCCAACGCG-3'   154

5' TTGTATATTATTTATTTTTTTTGGTTATGT-3' and   155
5' CTTAACACTCATAACTCACCAACACA-3'   156

5' ATATTCGTAGAAGGTAGCGCGGTC-3' and   157
5' AACGAACCCCTCCCGCG-3'   158

5' TAGTAAATATATTTGTAGAAGGTAGTGTGGTT-3' and   159
5' CAAAACAAACCCCTCCCACA-3'   160

5' ACGTAGACGTTTTATTAGGGTCGC-3' and   161
5' CCTCATCGTAACTACCCGCG-3'   162

5' TTTTGATGTAGATGTTTTATTAGGGTTGT-3' and   163
5' ACCACCTCATCATAACTACCCACA-3'   164

5' TTTTTGGCGTTAAAATGTCGTTC-3' and   165
5' TAAATAACTTCCCCCGCCG-3'   166

5' GGTTTTTTTGGTGTTAAAATGTTGTTT-3' and   167
5' CCACTAAATAACTTCCCCCACCA-3'   168

5' TCGTGGTCGGACGTCGTTC-3' and   169
5' CAACGTCTCCTTCGACTACACCG-3'   170

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5'GGTTGTTGTGGTTGGATGTTGTTT-3' and   171
5'CAACTACAACATCTCCTTCAACTACACCA-3'   172

5'GTTGTTAGGGGTATTCGGGTATC-3' and   173
5'CCCTAAAAAAACGTAAAAACGACG-3'   174

5
5'TGTTGTTAGGGGTATTTGGGTATT-3' and   175
5'TCCCCTAAAAAAACATAAAAACAACA-3'   176

5'TGGGTTCGTTTGGTAGTTCGC-3' and   177
5'CCTCCCTAACCGCCGCG-3'   178

10
5'GGTTGGGTTTGTTTGGTAGTTTGT-3' and   179
5'CCCACCTCCCTAACCACCACA-3'   180

5'CGACGATATTTTCGCGC-3' and   181
5'AAACCGACGAAACGCG-3'   182

15
5'GTATGGAGTGATGATATTTTTGTGT-3' and   183
5'ACCTAAAAACCAACAAAACACA-3'   184

5'TGTTTGTAGGCGGTTAGCGTC-3'and   185
5'ACGCACCCCTAAACGACCG-3'   186

20
5'TGGGTGTTTGTAGGTGGTTAGTGTT   187
5'AACATAACACACCCCTAAACAACCA-3'   188

5'GCGGTAGAGGCGGAGTC-3' and   189
5'CGAAATAAACTAACAAAAACCG-3'   190

25
5'ATTAGGTGGTAGAGGTGGAGTT-3' and   191
5'CCAAAATAAACTAACAAAAACCA-3'   192

5'TCGGTTTCGTTTTCGCGC-3' and   193
5'CCCAACATATCCGAAACGCG-3'   194

30
5'GTGTTTTGGTTTTGTTTTTGTGT-3' and   195
5'TCCCAACATATCCAAAACACA-3'   196

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5'TTACGGCGTTGAGGGACGTTC-3' and                    197
5'AAACGACCTACCGCGCATACG-3'                         198


5'GAGTTTTATGGTGTTGAGGGATGTTT-3' and               199
5'AAAAAAAAAAACAACCTACCACACATACA-3'                200


5'GTTTCGGGTTTGAGTTTCGC-3' and                     201
5'ATAATAACGATCCTCACGATAAACG-3'                     202


5'GGGTTTTGGGTTTGAGTTTTGT-3' and                   203
5'CCATAATAACAATCCTCACAATAAACA-3'                  204


5'GACGGTTTGGGGTAGTTTAGTAGC-3' and                 205
5'GACACCGCCATAACGACCG-3'                          206


5'GGATGGTTTGGGGTAGTTTAGTAGT-3' and                207
5'CTCCAACACCACCATAACAACCA-3'                       208
```

*Also included are modifications of the above sequences,
including SEQ ID NO:107 having the sequence TCAC at the 5' end; SEQ
ID NO:107 having the sequence CC added at the 5' end; SEQ ID NO:108
having the sequence 5'-TTATTAGAGGGTGGGGCGGATCGC-3'; SEQ ID NO:109
having the sequence 5'-GACCCCGAACCGCGACCGTAA-3'; SEQ IT NO:110 having
the sequence TGG added at the 5' end; and SEQ ID NO:111 having the
sequence TACC added at the 5' end. All of these modified primers
anneal at 65°C.

    Typically, the CpG-containing nucleic acid is in
the region of the promoter of a structural gene. For
example, the promoter region of tumor suppressor genes
have been identified as containing methylated CpG island.
The promoter region of tumor suppressor genes, including
p16, p15, VHL and E-cadherin, are typically the sequence
amplified by PCR in the method of the invention. Other
genes that have been shown by MSP as containing
methylated CpG neoplastic versus normal tissue include
estrogen receptor, MDGI, GST-pi, calcitonin, HIC-1,
endothelin B receptor, TIMP-2, 06-MGMT, and MLH1. Genes

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that were found by MSP to be methylated also include the
androgen receptor (e.g., methylated as X chromosome
inactivation), GFAP (methylated in some glioma cell lines
but also in normal tissue), and MSH2. Other genes in
5 which MSP primer were shown to distinguish between normal
unmethylated and methylated DNA include TGF-β1, TGF-β2,
p130, BRCA2, NF1, NF2, and TSG101.

Detection and identification of methylated CpG-
containing nucleic acid in the specimen may be indicative
10 of a cell proliferative disorder or neoplasia. Such
disorders include but are not limited to low grade
astrocytoma, anaplastic astrocytoma, glioblastoma,
medulloblastoma, colon cancer, lung cancer, renal cancer,
leukemia, breast cancer, prostate cancer, endometrial
15 cancer and neuroblastoma. Identification of methylated
CpG status is also useful for detection and diagnosis of
genomic imprinting, fragile X syndrome and X-chromosome
inactivation.

Using the method of the invention, the TIMP-2 gene
20 was identified as associated with or methylated in
neoplastic versus normal tissues.

The method of the invention now provides the basis
for a kit useful for the detection of a methylated CpG-
containing nucleic acid. The kit includes a carrier means
25 being compartmentalized to receive in close confinement
therein one or more containers. For example, a first
container contains a reagent which modifies unmethylated
cytosine, such as sodium bisulfite. A second container
contains primers for amplification of the CpG-containing
30 nucleic acid, for example, primers listed above as SEQ ID
NO:105-208.

The invention also provides a kit for the
detection of a methylated CpG-containing nucleic acid,

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wherein the kit includes: a) a reagent that modifies
unmethylated cytosine nucleotides; b) control nucleic
acid; c) primers for the amplification of unmethylated
CpG-containing nucleic acid; d) primers for the
5 amplification of methylated CpG-containing nucleic acid;
and e) primers for the amplification of control nucleic
acid. The kit may further include nucleic acid
amplification buffer. Preferably, the reagent that
modifies unmethylated cytosine is bisulfite.

10   The kit of the invention is intended to provide
the reagents necessary to perform chemical modification
and PCR amplification of DNA samples to determine their
methylation status. The primer sets included in the kit
include a set that anneals to unmethylated DNA that has
15 undergone a chemical modification; a set that anneals to
methylated DNA that has undergone a chemical
modification; and a primer set that serves as a control
for the efficiency of chemical modification. The control
primer set should anneal to any DNA (unmethylated or
20 methylated) that has not undergone chemical methylation.
In the case of incomplete chemical modification (up to
about 50%), data interpretation can still proceed.

The above disclosure generally describes the
present invention. A more complete understanding can be
25 obtained by reference to the following specific examples
which are provided herein for purposes of illustration
only and are not intended to limit the scope of the
invention.

Example 1

30   **DNA and Cell Lines.** Genomic DNA was obtained from
cell lines, primary tumors and normal tissue as described
(Merlo, *et al., Nature Medicine*, 1:686, 1995; Herman, *et*

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*al., Cancer Research, 56:722, 1996; Graff, et al., Cancer
Research, 55:5195, 1995).* The renal carcinoma cell line
was kindly provided by Dr. Michael Lehrman of the
National Cancer Institute, Bethesda, MD.

5   **Bisulfite Modification.** 1 µg of DNA in a volume of
50 µl was denatured by NaOH (final 0.2M) for 10 minutes
at 37 °C.   For samples with nanogram quantities of human
DNA, 1 µg of salmon sperm DNA (Sigma) was added as
carrier prior to modification. 30 µL of 10mM hydroquinone
10 (Sigma) and 520 µL of 3 M sodium bisulfite (Sigma) pH5,
both freshly prepared, were added, mixed, and samples
were incubated under mineral oil at 50 °C for 16 hours.
Modified DNA was purified using the Wizard™ DNA
purification resin according to the manufacturer
15 (Promega), and eluted into 50 µL of water. Modification
was completed by NaOH (final 0.3M) treatment for 5
minutes at room temperature, followed by ethanol
precipitation.

         Genomic Sequencing. Genomic sequencing of
20 bisulfite modified DNA was accomplished using the solid-
phase DNA sequencing approach (Myohanen, *et al., DNA
Seq.,* 5:1, 1994). 100 ng of bisulfite modified DNA was
amplified with *p16* gene specific primer 5'-
TTTTTAGAGGATTTGAGGGATAGG-3' (sense) (SEQ ID NO:209) and
25 5'-CTACCTAATTCCAATTCCCCTACA-3' (anti-sense) (SEQ ID
NO:210). PCR conditions were as follows: 96 °C for 3
minutes, 80 °C for 3 minutes, 1 U of *Taq* polymerase (BRL)
was added, followed by 35 cycles of 96 °C for 20 seconds,
56 °C for 20 seconds, 72 °C for 90 seconds, followed by 5
30 minutes at 72 °C. The PCR mixture contained 1X buffer
(BRL) with 1.5mM $MgCl_2$, 20 pmols of each primer and 0.2 mM
dNTPs. To obtain products for sequencing, a second round

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of PCR was performed with 5 pmols of nested primers. In
this reaction, the sense primer,

     5'-<u>GTTTTCCCAGTCACGAC</u>AGTATTAGGAGGAAGAAAGAGGAG-3' (SEQ
ID NO:211), contains M13-40 sequence (underlined)
5 introduced as a site to initiate sequencing, and the
anti-sense primer

     5'-TCCAATTCCCCTACAAACTTC-3" (SEQ ID NO:212) is
biotinylated to facilitate purification of the product
prior to sequencing. PCR was performed as above, for 32
10 cycles with 2.5 mM $MgCl_2$. All primers for genomic
sequencing were designed to avoid any CpGs in the
sequence. Biotinylated PCR products were purified using
streptavidin coated magnetic beads (Dynal AB, Norway),
and sequencing reactions performed with Sequenase™ and
15 M13-40 sequencing primer under conditions specified by
the manufacturer (USB).

     PCR Amplification. Primer pairs described in Table
1 were purchased from Life Technologies. The PCR mixture
contained 1X PCR buffer (16.6 mM ammonium sulfate, 67mM
20 TRIS pH 8.8, 6.7 mM $MgCl_2$, and 10 mM β-mercaptoethanol),
dNTPs (each at 1.25mM), primers (300 ng/reaction each),
and bisulfite-modified DNA (~50ng) or unmodified DNA (50-
100ng) in a final volume of 50 μL. PCR specific for
unmodified DNA also included 5% dimethylsulfoxide.
25 Reactions were hot started at 95 °C for 5 minutes prior
to the addition of 1.25 units of *Taq* polymerase (BRL).
Amplification was carried out on a Hybaid OmniGene
temperature cycler for 35 cycles (30 seconds at 95°C, 30
seconds at the annealing temperature listed in Table 1,
30 and 30 seconds at 72 °C), followed by a final 4 minute
extension at 72 °C. Controls without DNA were performed
for each set of PCR reactions. 10 μL of each PCR reaction
was directly loaded onto non-denaturing 6-8%

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polyacrylamide gels, stained with ethidium bromide, and
directly visualized under UV illumination.

Restriction Analysis. 10 µL of the 50 µL PCR
reaction was digested with 10 units of *BstUI* (New England
5 Biolabs) for 4 hours according to conditions specified by
the manufacturer. Restriction digests were ethanol
precipitated prior to gel analysis.

Example 2

An initial study was required to validate the
10 strategy for MSP for providing assessment of the
methylation status of CpG islands. The p16 tumor
suppressor (Merlo, *et al., supra;* Herman, *et al., Cancer
Research,* 55:4525, 1995; Gonzalez-Zulueta, *et al., Cancer
Res.,* 55:4531, 1995,27) which has been documented to have
15 hypermethylation of a 5' CpG island is associated with
complete loss of gene expression in many cancer types,
was used as an exemplary gene to determine whether the
density of methylation, in key regions to be tested, was
great enough to facilitate the primer design disclosed
20 herein. Other than for CpG sites located in recognition
sequences for methylation-sensitive enzymes, the density
of methylation and its correlation to transcriptional
silencing had not yet been established. The genomic
sequencing technique was therefore employed to explore
25 this relationship.

Figure 1 shows genomic sequencing of p16. The
sequence shown has the most 5' region at the bottom of
the gel, beginning at +175 in relation to a major
transcriptional start site (Hara, *et al., Mol. Cell
30 Biol.,* 16:859, 1996). All cytosines in the unmethylated
cell line H249 have been converted to thymidine, while
all C's in CpG dinucleotides in the methylated cell H157

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remains as C, indicating methylation.] enclosed a *BstUI*
site which is at -59 in relation to the transnational
start site in Genbank sequence U12818 (Hussussian, *et
al., Nat. Genet.*, 8:15, 1994), but which is incorrectly
5   identified as CGCA in sequence X94154 (Hara, *et al.,
supra*). This CGCG site represents the 3' location of the
sense primer used for *p16* MSP.

As has been found for other CpG islands examined
in this manner (Myohanen, *et al., supra*; Park, *et al.,
10 Mol. Cell Biol.*, 14:7975, 1994; Reeben, *et al., Gene*,
157:325, 1995), the CpG island of *p16* was completely
unmethylated in those cell lines and normal tissues
previously found to be unmethylated by Southern analysis
(Fig. 1)(Merlo, *et al., supra*; Herman, *et al., supra*).
15 However, it was extensively methylated in cancer cell
lines shown to be methylated by Southern analysis (Fig.
1). In fact, all cytosines within CpG dinucloetides in
this region were completely methylated in the cancers
lacking *p16* transcription. This marked difference in
20 sequence following bisulfite treatment suggested that the
method of the invention for specific amplification of
either methylated or unmethylated alleles was useful for
identification of methylation patterns in a DNA sample.

Primers were designed to discriminate between
25 methylated and unmethylated alleles following bisulfite
treatment, and to discriminate between DNA modified by
bisulfite and that which had not been modified. To
accomplish this, primer sequences were chosen for regions
containing frequent cytosines (to distinguish unmodified
30 from modified DNA), and CpG pairs near the 3' end of the
primers (to provide maximal discrimination in the PCR
reaction between methylated and unmethylated DNA). Since
the two strands of DNA are no longer complementary after

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bisulfite treatment, primers can be designed for either
modified strand. For convenience, primers were designed
for the sense strand. The fragment of DNA to be amplified
was intentionally small, to allow the assessment of

5   methylation patterns in a limited region and to
facilitate the application of this technique to samples,
such as paraffin blocks, where amplification of larger
fragments is not possible. In Table 1, primer sequences
are shown for all genes tested, emphasizing the

10   differences in sequence between the three types of DNA
which are exploited for the specificity of MSP. The
multiple mismatches in these primers which are specific
for these different types of DNA suggest that each primer
set should provide amplification only from the intended

15   template.

        The primers designed for *p16* were tested with DNA
from cancer cell lines and normal tissues for which the
methylation status had previously been defined by
Southern analysis (Merlo, *et al.*, *supra*; Herman, *et al.*,

20   *supra*).

        Figure 2, panels A-D, show polyacrylamide gels
with the Methylation Specific PCR products of p16. Primer
sets used for amplification are designated as
unmethylated (U), methylated (M), or unmodified/wild-type

25   (W).* designates the molecular weight marker pBR322-*MspI*
digest. Panel A shows amplification of bisulfite-treated
DNA from cancer cell lines and normal lymphocytes, and
untreated DNA (from cell line H249). Panel B shows mixing
of various amount of H157 DNA with 1 µg of H249 DNA prior

30   to bisulfite treatment to assess the detection
sensitivity of MSP for methylated alleles. Modified DNA
from a primary lung cancer sample and normal lung are
also shown. Panel C shows amplification with the p16-U2

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(U) primers, and p16-M2 (M) described in Table 1. Panel D
shows the amplified *p16* products of panel C restricted
with *BstUI*(+) or not restricted (-).

In all cases, the primer set used confirmed the
5   methylation status determined by Southern analysis. For
example, lung cancer cell lines U1752 and H157, as well
other cell lines methylated at *p16*, amplified only with
the methylated primers (Fig. 2, panel A). DNA from normal
tissues (lymphocytes, lung, kidney, breast, and colon)
10 and the unmethylated lung cancer cell lines H209 and
H249, amplified only with unmethylated primers (examples
in Fig 2, panel A). PCR with these primers could be
performed with or without 5% DMSO. DNA not treated with
bisulfite (unmodified) failed to amplify with either set
15 of methylated or unmethylated specific primers, but
readily amplified with primers specific for the sequence
prior to modification (Fig. 2, panel A). DNA from the
cell line H157 after bisulfite treatment also produced a
weaker amplification with unmodified primers, suggesting
20 an incomplete bisulfite reaction. However, this
unmodified DNA, unlike partially restricted DNA in
previous PCR assays relying on methylation sensitive
restriction enzymes, is not recognized by the primers
specific for methylated DNA. It therefore does not
25 provide a false positive result or interfere with the
ability to distinguish methylated from unmethylated
alleles.

The sensitivity of MSP for detection of methylated
p16 alleles was assessed. DNA from methylated cell lines
30 was mixed with unmethylated DNA prior to bisulfite
treatment. 0.1% of methylated DNA (approximately 50 pg)
was consistently detected in an otherwise unmethylated
sample (Fig. 2, panel B). The sensitivity limit for the

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amount of input DNA was determined to be as little as 1
ng of human DNA, mixed with salmon sperm DNA as a carrier
detectable by MSP.

Fresh human tumor samples often contain normal and
5   tumor tissue, making the detection of changes specific
for the tumor difficult. However, the sensitivity of MSP
suggests it would be useful for primary tumors as well,
allowing for detection of aberrantly methylated alleles
even if they contribute relatively little to the overall
10   DNA in a sample. In each case, while normal tissues were
completely unmethylated, tumors determined to be
methylated at p16 by Southern analysis also contained
methylated DNA detected by MSP, in addition to some
unmethylated alleles (examples in Fig. 2, panel B). DNA
15   from paraffin-embedded tumors was also used, and allowed
the detection of methylated and unmethylated alleles in
these samples (Fig. 2, panel B). To confirm that these
results were not unique to this primer set, a second
downstream primer for p16 was used which would amplify a
20   slightly larger fragment (Table 1). This second set of
primers reproduced the results described above (Fig 2,
panel C), confirming the methylation status defined by
Southern blot analysis.

To further verify the specificity of the primers
25   for the methylated alleles and to check specific
cytosines for methylation within the region amplified,
the differences in sequence between methylated/modified
DNA and unmethylated/modified DNA were utilized.
Specifically, the BstUI recognition site, CGCG, will
30   remain CGCG if both C's are methylated after bisulfite
treatment and amplification, but will become TGTG if
unmethylated. Digestion of the amplified products with
BstUI distinguishes these two products. Restriction of

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p16 amplified products illustrates this. Only unmodified
products and methylated/modified products, both of which
retain the CGCG site, were cleaved by *BstUI*, while
products amplified with unmethylated/modified primers
5 failed to be cleaved (Fig. 2, panel D).

The primer sets discussed above were designed to
discriminate heavily methylated CpG islands from
unmethylated alleles. To do this, both the upper (sense)
and lower (antisense) primers contained CpG sites which
10 could produce methylation-dependent sequence differences
after bisulfite treatment. MSP might be employed to
examine more regional aspects of CpG island methylation.
To examine this, methylation-dependent differences in the
sequence of just one primer was tested to determine
15 whether it would still allow discrimination between
unmethylated and methylated *p16* alleles. The antisense
primer used for genomic sequencing, 5'-
CTACCTAATTCCAATTCCCCTACA-3' (SEQ ID NO:213), was also
used as the antisense primer, since the region recognized
20 by the primer contains no CpG sites, and was paired with
either a methylated or unmethylated sense primer (Table
1). Amplification of the 313 bp PCR product only occurred
with the unmethylated sense primer in H209 and H249
(unmethylated by Southern) and the methylated sense
25 primer in H157 and U1752 (methylated by Southern),
indicating that methylation of CpG sites within a defined
region can be recognized by specific primers and
distinguish between methylated and unmethylated alleles
(Fig. 2, panel E). Panel E shows results of testing for
30 regional methylation of CpG islands with MSP, using sense
primers p16-U2 (U) and p16-M2 (M), which are methylation
specific, and an antisense primer which is not
methylation specific.

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Example 3

The above experiments with p16 were extended to include 3 other genes transcriptionally silenced in human cancers by aberrant hypermethylation of 5' CpG islands.

5   Figure 3, panels A-E, show polyacrylamide gels of MSP products from analysis of several genes. Primer sets used for amplification are not designated as unmethylated (U), methylated (M), or unmodified/wild-type (W). * designates the molecular weight marker pBR322-*MspI* digest

10   and ** designates the 123bp molecular weight marker. All DNA samples were bisulfite treated except those designated untreated. Panel A shows the results from MSP for *p15*. Panel B shows the *p15* products restricted with *BstUI* (+) or not restricted (-). Panel C shows the

15   products of MSP for VHL. Panel D shows the VHL products restricted with *BstUI*(+) or not restricted (-). Panel E shows the products of MSP for *E-cadherin*.

The cyclin-dependent kinase inhibitor *p15* is aberrantly methylated in many leukemic cell lines and

20   primary leukemias (Herman, *et al.*, *supra*). For *p15*, MSP again verified the methylation status determined by Southern analysis. Thus, normal lymphocytes and cancer cell lines SW48 and U1752, all unmethylated by Southern analysis (Herman, *et al.*, *supra*), only amplified with the

25   unmethylated set of primers, while the lung cancer cell line H1618 and leukemia cell line KG1A amplified only with the methylated set of primers (Fig. 3, panel A), consistent with previous Southern analysis results (Herman, *et al.*, *supra*). The cell line Raji produced a

30   strong PCR product with methylated primers and a weaker band with unmethylated primers. This was the same result for methylation obtained previously by Southern analysis (Herman, *et al.*, *supra*). Non-cultured leukemia samples,

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like the primary tumors studied for p16, had
amplification with the methylated primer set as well as
the unmethylated set. This heterogeneity also matched
Southern analysis (Herman, et al., supra). Again, as for
5 p16, differential modification of BstUI restriction sites
in the amplified product of p15 was used to verify the
specific amplification by MSP (Fig. 3, panel B).
Amplified products using methylated primer sets from cell
lines H1618 and Raji or unmodified primer sets, were
10 completely cleaved by BstUI, while unmethylated amplified
products did not cleave. Primary AML samples, which again
only demonstrated cleavage in the methylated product, had
less complete cleavage. This suggests a heterogeneity in
methylation, arising because in some alleles, many CpG
15 sites within the primer sequences area are methylated
enough to allow the methylation specific primers to
amplify this region, while other CpG sites are not
completely methylated.

Aberrant CpG island promoter region methylation is
20 associated with inactivation of the VHL tumor suppressor
gene in approximately 20% of clear renal carcinomas
(Herman, et al., Proc. Natl. Acad. Sci. USA, 91:9700,
1994). This event, like mutations for VHL (Gnarra, et
al., Nature Genetics, 7:85, 1994), is restricted to clear
25 renal cancers (Herman, et al., supra). Primers designed
for the VHL sequence were used to study DNA from the
renal cell cancer line RFX393 which is methylated at VHL
by Southern analysis, and the lung cancer cell line U1752
which is unmethylated at this locus (Herman, et al.,
30 supra). In each case, the methylation status of VHL
determined by MSP confirmed that found by Southern
analysis (Fig. 3, panel C), and BstUI restriction site

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analysis validated the PCR product specificity (Fig. 3,
panel D).

The expression of the invasion/metastasis
suppressor gene, *E-cadherin*, is often silenced by
5   aberrant methylation of the 5' promoter in breast,
prostate, and many other carcinomas (Graff, *et al.*,
supra; Yoshira, *et al.*, *Proc. Natl. Acad. Sci. USA*,
92:7416, 1995). Primers were designed for the *E-cadherin*
promoter region to test the use of MSP for this gene. In
10 each case, MSP analysis paralleled Southern blot analysis
for the methylation status of the gene (Graff, *et al.*,
supra). The breast cancer cell lines MDA-MB-231, HS578t,
and the prostate cancer cell lines DuPro and TSUPrI, all
heavily methylated by Southern, displayed prominent
15 methylation. MCF7, T47D, PC-3, and LNCaP, all
unmethylated by Southern, showed no evidence for
methylation in the sensitive MSP assay (Fig. 3, panel E).
MSP analysis revealed the presence of unmethylated
alleles in Hs578t, TSUPrI and DuPro consistent with a low
20 percentage of unmethylated alleles in these cell lines
previously detected by Southern analysis (Graff, *et al.*,
supra). *BstUI* restriction analysis again confirmed the
specificity of the PCR amplification.

Table 1 PCR primers used for Methylation Specific PCR

Primer Set	Sense primer* (5'-3')	Antisense primer* (5'-3')	Size (bp)	Anneal temp.	Genomic Position†
p16-Wt	CAGAGGGTGGGGCGACCGC	CGGGCCGCGGCCGTGG	140	65°C	+171
p16-M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65°C	+167
p16-U	TTATTAGAGGGTGGGGTGGATTGT	C AACCCCAAACCACAACCATAA	151	65°C	+167
p16-M2	TTATTAGAGGGTGGGGCGGATCGC	CCACCTAAATCGACCTCCGACCG	234	65°C	+167
p16-U2	TTATTAGAGGGTGGGGTGGATTGT	CCACCTAAATCAACCTCCAACCA	234	60°C	+167
p15-W	CGCACCCTGCGGCCAGA	AGTGGCCGAGCGGCCGG	137	65°C	+46
p15-M	GCGTTCGTATTTTGCGGTT	CGTACAATAACCGAACGACCGA	148	60°C	+40
p15-U	TGTGATGTGTTTGTATTTTGTGGTT	CCATACAATAACCAAACAACCAA	154	60°C	+34
VHL-M	TGGAGGATTTTTTTGCGTACGC	GAACCGAACGCCGCGAA	158	60°C	-116
VHL-U	GTTGGGAGGATTTTTTTTGTGTATGT	CCCAAACCAAACACCACACAAAA	165	60°C	-118
Ecad-M	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	116	57°C	+205
Ecad-U	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACACA	97	53°C	-210

*Sequence differences between modified primers and unmodified DNA are boldface, and differences between methylated/modified and unmethylated/modified are underlined.
†Primers were placed near the transcriptional strat site. Genomic position is the location of the 5' nucleotide of the sense primer in relation to the major transcriptional start site defined in the following references and Genbank accession numbers: p16(most 3' site) X94154 (E. Hara, et al., Mol. Cell Biol., 16:859, 1996).

p15 S75756 (J. Jen. et al., Cancer Res., 54: 6353 1994), VHL U19763 (I. Kuzmin, et al., Oncogene, 10:2185 1995), and E-cadherin L34545 (M. J. Bussemakers.

et al., Biochem.Biophys. Res. Commun..203: 1284 1994).

†W represents unmodified, or wild-type primers, M represents methylated-specific primers, and U represents unmethylated-specific primers.

(SEQ ID NO:105-128)

Table 2

Gene		Sense Primer	Antisense Primer	PCR Product	Temp	Genbank#	Position
Androgen Receptor	Me	5'gggtttaggtattagaggtcgc	5'accaaataacctataaacctctacg	~210bp	55°C	M27423	189
	Un	5'gggtttaggtattagaggttgt	5'accaaataacctataaacctctaca	~210bp	55°C	M27423	189
Estrogen Receptor	Me	5'gtgtatttggatagtagttcgtc	5'cgtaaaaaaccgatctaaccgtaaa	118bp	57°C	X62462	3127
	Un	5'ggtgtatttggatagtagttgt	5'ccataaaaaaccaatctaaccaaca	120bp	57°C	X62462	3126
MDGI	Me	5'tttatttcgggagtcggggc	5'aacgtacaaaaactccgacgacg	105bp	60°C	U57623	1134
	Un	5'tttttatttgggagttggggt	5'aaacatacaaaaactccaacaaca	113bp	60°C	U57623	1131
GS	Me	5'ttcggggtgtagcggtcgtc	5'gccccaatactaaatcacgacg	91bp	60°C	X08058	1082
	Un	5'gatgtttggggtgtagtggttgtt	5'ccaccccaatactaaaatcaacaaca	97bp	60°C	X08058	1078
Calcitonin	Me	5'cgggaataagagtagtcgttggc	5'caaaaccctataaaacgacgacg	84bp	60°C	X15943	1801
	Un	5'ggtgggaataagagtagttgttggt	5'ccaaaacccctataaaaacaacaacaca	87bp	60°C	X15943	1799
HIC-1	Me	5'gtcgtagtcggttgtgcgtc	5'gatacccgcgcctaacgccg	259bp	60°C		
	Un	5'gggtagttagttggttgtgtt	5'cacaatacccaccccctaacacca	264bp	60°C		

Endo-theli	Me	5'gtatattattatttttttggttacgc	5'tcataactcgccaacgcg	92bp	55°C	D13162	969
	Un	5'ttgtatattatttttttttggttatgt	5'cttaacactcataactcaccaccaca	102bp	55°C	D13162	967
TIMP-2	Me	5'atattcgtagaaggtagcgcggtc	5'aacgaacccctccgcg	190bp	59°C	U44381	2254
	Un	5'tagtaaatatatttgagaaggtagtggtt	5'caaaacaaaccccctcccaca	201bp	59°C	U44381	2246
5 MLH1-a	Me	5'acgtagacgtttattagggtcgc	5'cctcatcgtaactacccgcg	115bp	60°C	U26559	218
	Un	5'tttgatgtgagatgtttattagggttgt	5'accacctcatcataactacccaca	124bp	60°C	U26559	213
MLH1-b	Me	5'ttttttggttaaatgtcgttc	5'taaataacttccccgccg	91bp	60°C	U26559	920
	Un	5'ggttttttggtgttggtaaatgttgtt	5'ccactaaataacttcccccacca	99bp	60°C	U26559	916
MSH2	Me	5'tcgtgtcggacgtcgttc	5'caacgtctccttcgactacaccg	132	60°C	U41206	128
	Un	5'ggttgttgttggtggtgttgttt	5'caactacaacatctccttcaactacacca	143	60°C	U41206	123
10 GFAP	Me	5'gttgttaggggtattcgggtatc	5'ccctaaaaacgtaaaaacgacg	140bp	60°C	M67446	2092
	Un	5'tgttgttaggggtatttgggtatt	5'tcccctaaaaaaacataaaaacaaca	143bp	60°C	M67446	2091
TGFβ1	Me	5'tgggttcgtttggtagttcgc	5'cctccctaaccgccgcg	124bp	57°C	U51139	1223
	Un	5'ggttgggtttgttgttggtagtttgt	5'cccacctccctaaccaccacca	131bp	57°C	U51139	1220

TGFβ2	Me	5'cgacgatatttcgcgc	5'aaaccgacgaaacgcg	97bp	57°C	U52240	72
	Un	5'gtatggagtgatgatattttgtgt	5'acctaaaaaccaacaaaacaca	111bp	57°C	U52240	64
p130	Me	5'tgtttgtaggcggttagcgtc	5'acgcacccctaaacgaccg	135bp	55°C	U53220	2076
	Un	5'tgggtgtttgtaggtggttagtgtt	5'aacataacacaccccctaaacacca	145bp	55°C	U53220	2072
BRCA2	Me	5'gcggtagaggcgggagtc	5'cgaaataaactaacaaaaaccg	136bp	55°C	X95151	292
	Un	5'attaggtggtagaggtggagtt	5'ccaaaataaactaacaaaaaca	142bp	55°C	X95151	287
O⁶-MGMT	Me	5'tcggtttcgtttcgcgc	5'cccaacatatccgaaacgcg	125bp	60°C	S52289	114
	Un	5'gtgttttggttttgtttttgt	5'tcccaacatatccaaaacaca	131bp	60°C	S52289	109
NF1	Me	5'ttacggcgttgaggacgttc	5'aaacgacctaccgcgcatacg	121bp	60°C	U17084	3336
	Un	5'gagtttatggtgttgagggatgttt	5'aaaaaaaaaacaacctaccaccacataca	134bp	60°C	U17084	3331
NF	Me	5'gtttcgggtttgagtttcgc	5'ataataacgatcctcacgataaacg	109bp	55°C	L27131	38
	Un	5'ggggtttgggtttgagtttgt	5'ccataataacaatcctcacaataaaca	113bp	55°C	L27131	36
TSG101	Me	5'gacggtttggggtagtttagtagc	5'gacaccgccataacgaccg	80bp	60°C	U82130	23
	Un	5'ggatggtttggggtagtttagtagt	5'ctccaacaccaccataaacaacca	85bp	60°C	U82130	22

(SEQ ID NO:25-104)

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Example 4

Indirect *in situ* p16 Methylation. Indirect *in situ*
PCR (indirect *IS*-PCR) describes a technique whereby the
5 amplicon is produced by thermal cycling without label
incorporation. At the end of the cycling reaction, the
amplified product is detected by standard *in situ*
hybridization using a labeled probe.

Sample Preparation and Pretreatment. Paraffin-
10 embedded formalin fixed HN12 cells were deparaffinized in
xylene for 5 minutes, followed by two 5 minute rinses in
100% ethanol. Slides were air dried, and then treated
with Proteinase K(10μg/ml in 50mM Tris) for 30 minutes at
37°C under a coverslip. Slides were rinsed in distilled
15 water and placed in 0.2N NaOH for 10 minutes at 37°C, then
placed in 3M Sodium bisulfite containing hydroxyquinone.
The solution was layered with mineral oil and left at 50°C
for 16 hours. The oil was removed, and the slides were
rinsed in water and placed in 0.3M NaOH for 5 minutes at
20 room temperature. Slides were then dehydrated in 70%,
80%, and 100% ethanol and air dried.

Indirect *IS*-PCR Protocol. The PCR mixture
contained 1xPCR buffer (16.6mM ammonium sulfate/67mM
Tris, pH 8.8/6.7mM MgCl$_2$/10mM 2-mercaptoethanol), dNTPs
25 (2.5 lambda of 25mM mix), 300ng of each primer, and 2.5
Units of *Taq* Polymerase per 50μl reaction. This mixture
was "hot started" at 95°C for 5 minutes, then added
directly to the slides which were prewarmed to 80°C with a
GeneComb in place. PCR was performed on a Hybaid
30 Omnigene Cycler with an *in situ* module. Thirty cycles of
90°C for 30 seconds, 58°C for 45 seconds, and 70°C for 45
seconds were performed with a calibration number of 50.
After the PCR, slides were immediately rinsed once in

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distilled water, followed by dehydration in 70%, 80%, and
100% ethanol and air dried.

**Indirect *IS*-PCR *In Situ* Hybridization and
Fluorescence Detection.** Following the PCR, the specimen

5 was fixed briefly to maintain the localization of the PCR
product. This was accomplished by fixing the sections in
100% ethanol for 15 minutes and then allowing them to air
dry before application of the probe. A methylated p16
PCR product was generated and labeled with digoxigenin,

10 and used as a labeled probe for *in situ* hybridization.
The digoxigenin labeled probe was suspended in a
hybridization buffer containing 2xSSC/10% Dextran
Sulfate/50% Formamide for a final concentration of
10ng/ul. 10.0μl(100.0ng of probe) of the hybridization

15 mixture was placed on each sample, covered with a glass
coverslip and sealed with rubber cement. The cell
preparations were then incubated at 95°C for 5 minutes and
hybridized at 37°C overnight in a humidified chamber.

Following hybridization, the HN12 samples were

20 post-washed in 2xSSC (salt sodium citrate) at room
temperature for five minutes and placed in 1xPBD
(phosphate buffered detergent) at room temperature prior
to immunological detections.

All hybridized HN12 cell samples were

25 immunocytochemically stained with fluorescein labeled
avidin (Vector Laboratories, CA). Sixty microliters of
detection reagent, consisting of fluorescein labeled
avidin (10ug/ml), 1XPBS, 5% powdered dry milk, and 0.02%
sodium azide was applied to the slide under a plastic

30 coverslip and incubated at 37°C for five minutes in a
humidified chamber. The slides were washed in 1XPBD
several times and counterstained with propidium iodide
(0.3ug/ml) in antifade solution.

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For fluorescence microscopy, a Zeiss Axiophot 20
epi-fluorescence microscope (Zeiss, West Germany)
equipped with a 100-Watt mercury-arc lamp, a 100X
Plan-Neofluar oil immersion objective (Zeiss, West

5   Germany), a Zeiss MC-100 camera (Zeiss, West Germany),
and the appropriate filter sets for fluorescein (FITC)/PI
fluorescence (Chroma, VT) were utilized. Image analysis
and record keeping was performed using an OIS (Oncor
Instrument Systems) 3CCD Cooled Camera System and OIS

10 2.01 Image Analysis Software.

The negative PCR control HN12 cell sample was
processed using an initial bisulfite reaction to modify
the DNA, followed by PCR amplification using primers
specific for the methylated DNA of the promoter regions

15 of the p16 gene, and *in situ* hybridization. The negative
control displayed no visible signal as expected, since
the PCR reaction was incomplete.

P16 methylated HN12 cells were subjected to the
bisulfite modification, PCR amplification using primers

20 specific for the methylated DNA of the promoter regions
of the p16 gene, and *in situ* hybridization using the
labeled probe. Fluorescent signals specific for the
amplified, localized product of the methylated p16
promoter regions were visible in the cells.

25 P16 methylated HN12 cells were subjected to the
bisulfite modification, PCR amplification using primers
specific for the unmethylated DNA of the promoter regions
of the p16 gene, and *in situ* hybridization using the
labeled probe. No fluorescent signals were visible in

30 the cells.

Example 5

Testing the Use of MSP for Methylation

Changes in Sputum Samples. MSP has been tested to detect

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methylation changes in DNA from sputum samples.   The
samples tested included the primers for p16 (Table 1; SEQ
ID NOS:105-110) and DNA extracted from sputum samples
from two patients known to have lung cancer.   The
5 unmethylated alleles for p16 were detected, however,
methylated alleles were not detected. It appears that
since the MSP worked with the non-methylated alleles,
that the patient's tumors did not have methylated p16.
To test this hypothesis, one sputum sample was spiked
10 with cells from the established lung cancer cell line
Calu 3, which has a hypermethylated p16 gene.   These p16
methylated alleles were easily detected in this mixed
DNA.

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Example 6

MSP for detection of hMLH1 and hMSH2

hypermethylation. The hMLH1 and hMSH2 genes encode for
mismatch repair proteins, and mutations in each can cause
5   inherited forms of colon cancer marked by microsatellite
instability. Some 20% of patients with non-inherited
colon cancer also have tumors with the microsatellite
instability phenotype. The precise mechanism causing
these latter tumors is not clear. Kane *et al.* (*Cancer*
10 *Research*, 57:808, 1997) have reported in small series,
that tumors from patients with the sporadic form of colon
cancers with microsatellite instability have
hypermethylation of the promoter of the hMLH1 gene. MSP,
employing the primer set and conditions for hMSH2 and
15 hMLH1 (Table 2; SEQ ID NO:161-172), was used to follow up
on this observation in a larger series of tumors. In
some 14 patients with such tumors, 85% have
hypermethylation of hMLH1 while only 5% of 21 colon
tumors without microsatellite instability show this
20 change. No hypermethylation of hMSH2 was seen in either
group. The data indicate that 15% or so of all patients
with colon cancer, who are known to have tumors with
microsatellite instability, have hypermethylation of the
hMLH1 gene in tumor DNA, and that transcriptional
25 silencing associated with this change is the cause of the
genetic instability in this setting.

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Example 7

MSP studies of the Tissue Inhibitor of Metalloproteinase II (TIMP 2) gene. TIMP2 is a member of a family of metalloproteinase inhibitors which are

5 necessary to limit the invasive potential of multiple cell types. Using the MSP primers and conditions for this gene (Table 2; SEQ ID NOS:157-160), hypermethylation of the TIMP2 promoter was found in approximately 50% of primary colon cancer. This change, and the associated

10 loss of expression for this gene, could be a key factor in increasing the invasive potential for the colon cancers involved.

Example 8

MSP Studies of the TGF-beta Receptor I and II

15 **genes.** MSP primers and conditions previously provided (Table 2; SEQ ID NOS:177-184) have been successfully employed for TGF-beta receptor I and II genes, and no evidence for promoter hypermethylation in tumors has been found to date.

20

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

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What is claimed is:

1.   A method for detecting a methylated CpG-
        containing nucleic acid comprising:
        contacting a nucleic acid-containing specimen
        with an agent that modifies unmethylated
5   cytosine, amplifying the CpG-containing
        nucleic acid in the specimen by means of CpG-
        specific oligonucleotide primers, wherein
        the oligonucleotide primers distinguish
10   between modified methylated and non-
        methylated nucleic acid and detecting the
        methylated nucleic acid.

2.   The method of claim 1, wherein the amplifying
        step is the polymerase chain reaction (PCR).

15   3. The method of claim 1, wherein the modifying
        agent is bisulfite.

4.   The method of claim 1, wherein cytosine is
        modified to uracil.

5.   The method of claim 1, wherein the CpG-
20   containing nucleic acid is in a promoter
        region.

6.   The method of claim 5, wherein the promoter
        is a tumor suppressor gene promoter.

7.   The method of claim 6, wherein the tumor
25   suppressor gene is selected from the group
        consisting of p16, p15, E-cadherin, and VHL.

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8.   The method of claim 1, wherein the CpG-
      containing nucleic acid encodes a protein
      selected from the group consisting of
      androgen receptor, estrogen receptor, TGF-
      beta1, TGF-beta2, p130, BRCA2, NF1, NF2,
      TSG101, MDGI, GST-pi, calcitonin, HIC-1,
      endothelin B receptor, TIMP-2, 06-MGMT, MLH1,
      MSH2, and GFAP.

9.   The method of claim 1, wherein the specimen
      is selected from the group consisting of
      brain, colon, urogenital, lung, renal,
      hematopoietic, breast, thymus, testis,
      ovarian, and uterine tissue or serum, urine,
      saliva, cerebrospinal fluid, sputum,
      ejaculate, blood, and stool.

10.   The method of claim 1, further comprising
      contacting the nucleic acid with a
      methylation sensitive restriction
      endonuclease.

11.   The method of claim 10, wherein the
      restriction endonuclease is selected from the
      group consisting of *Msp*I, *Hpa*II, *BssH*II,
      *Bst*UI and *Not*I.

12.   The method of claim 1, wherein the presence
      of methylated CpG-containing nucleic acid in
      the specimen is indicative of a cell
      proliferative disorder.

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13.   The method of claim 12, wherein the disorder
        is selected from the group consisting of low
        grade astrocytoma, anaplastic astrocytoma,
        glioblastoma, medulloblastoma, colon cancer,
        lung cancer, renal cancer, leukemia, breast
        cancer, prostate cancer, endometrial cancer
        and neuroblastoma.

14.   The method of claim 1, wherein the primer
        hybridizes with a target polynucleotide
        sequence having the sequence selected from
        the group consisting of SEQ ID NO:1-103 and
        SEQ ID NO:104.

15.   The method of claim 1, wherein the primers
        are selected from the group consisting of SEQ
        ID NO:105-207 and SEQ ID NO:208.

16.   A kit useful for the detection of a
        methylated CpG-containing nucleic acid
        comprising carrier means being
        compartmentalized to receive in close
        confinement therein one or more containers
        comprising a first container containing a
        reagent which modifies unmethylated cytosine.

17.   The kit of claim 16, wherein the kit further
        comprises a second container containing
        primers for amplification of the CpG-
        containing nucleic acid.

18.   The kit of claim 16, wherein the reagent is
        bisulfite.

19.   The kit of claim 16, wherein cytosine is modified to uracil.

20.   The kit of claim 17, wherein the primer hybridizes with a target polynucleotide sequence having the sequence selected from the group consisting of SEQ ID NO:1-103 and SEQ ID NO:104.

21.   The kit of claim 17, wherein the primers are selected from the group consisting of SEQ ID NO:105-207 and SEQ ID NO:208.

22.   A kit for the detection of methylated CpG-containing nucleic acid from a sample comprising:
        a) a reagent that modifies unmethylated cytosine nucleotides;
        b) control nucleic acid;
        c) primers for the amplification of unmethylated CpG-containing nucleic acid;
        d) primers for the amplification of methylated CpG-containing nucleic acid; and
        e) primers for the amplification of control nucleic acid.

23.   The kit of claim 22, further comprising nucleic acid amplification   buffer.

24.   The kit of claim 22, wherein the reagent that modifies unmethylated cytosine is bisulfite.

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25.   The kit of claim 22, wherein primers
        hybridize with a target polynucleotide
        sequence having the sequence selected from
        the group consisting of SEQ ID NO:1-103 and
5   SEQ ID NO:104.

26.   The kit of claim 22, wherein the primers are
        selected from the group consisting of SEQ ID
        NO:105-207 and SEQ ID NO:208.

10

27.   Isolated oligonucleotide primer(s) for
        detection of a methylated CpG-containing
        nucleic acid wherein the primer hybridizes
        with a target polynucleotide sequence having
15   the sequence selected from the group
        consisting of SEQ ID NO:1-103 and SEQ ID
        NO:104.

28.   The primers of claim 27, wherein the primer
        pairs are SEQ ID NO:105-207 and SEQ ID
20   NO:208, in sequential pairs.

29.   A method for detecting a cell proliferative
        disorder associated with methylation of CpG
        islands in TIMP-2 nucleic acid a subject
        comprising:
25   contacting a target nucleic acid in a sample of
tissue or biological fluid from the subject with a reagent
which detects TIMP-2 wherein the reagent detects
methylation of TIMP-2 when the target nucleic acid is DNA,
and wherein the reagent detects the level of TIMP-2 RNA
30 when the target nucleic acid is RNA; and

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detecting TIMP-2 target nucleic acid,
respectively, wherein hypermethylation of the promoter of
DNA, respectively, or decreased levels of TIMP-2 RNA,
respectively, as compared with the level of TIMP-2 RNA
5 respectively, in a normal cell, is indicative of a TIMP-
2-associated cell proliferative disorder.

30.   The method of claim 29, wherein the reagent
        is a nucleic acid probe.

31.   The method of claim 30, wherein the probe is
10   detectably labeled.

32.   The method of claim 31, wherein the label is
        selected from the group consisting of
        radioisotope, a bioluminescent compound, a
        chemiluminescent compound, a fluorescent
15   compound, a metal chelate, and an enzyme.

33.   The method of claim 29, wherein the reagent
        which detects methylation is a restriction
        endonuclease.

34.   The method of claim 33, wherein the
20   restriction endonuclease is methylation
        sensitive.

35.   The method of claim 34, wherein the
        restriction endonuclease is selected from the
        group consisting of *Msp1*, *Hpa*II and *Bss*HII.

25 36.   The method of claim 29, wherein the
        biological fluid is selected from the group
        consisting of serum, urine, saliva,
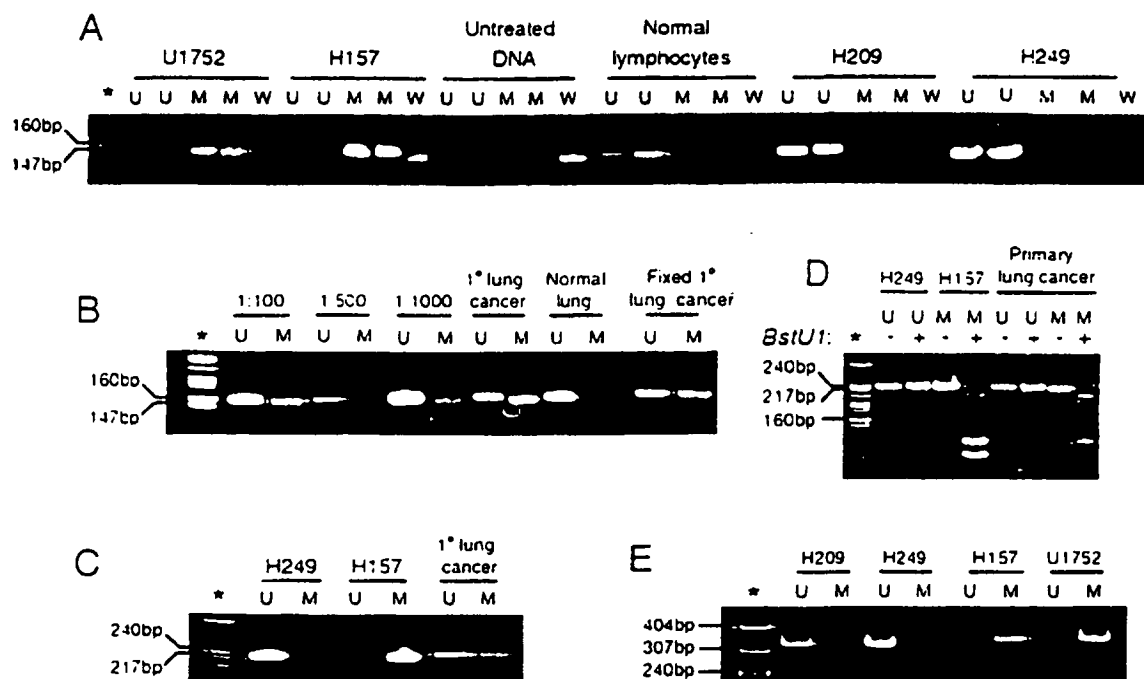
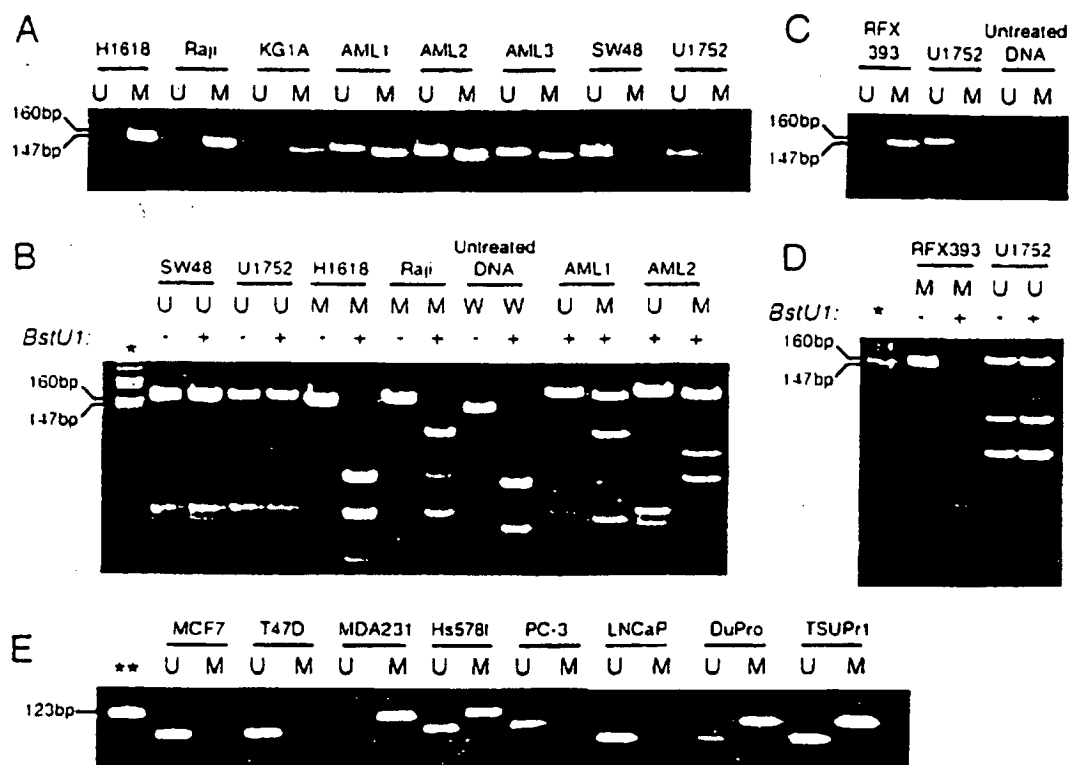cerebrospinal fluid, sputum, ejaculate, blood, and stool.
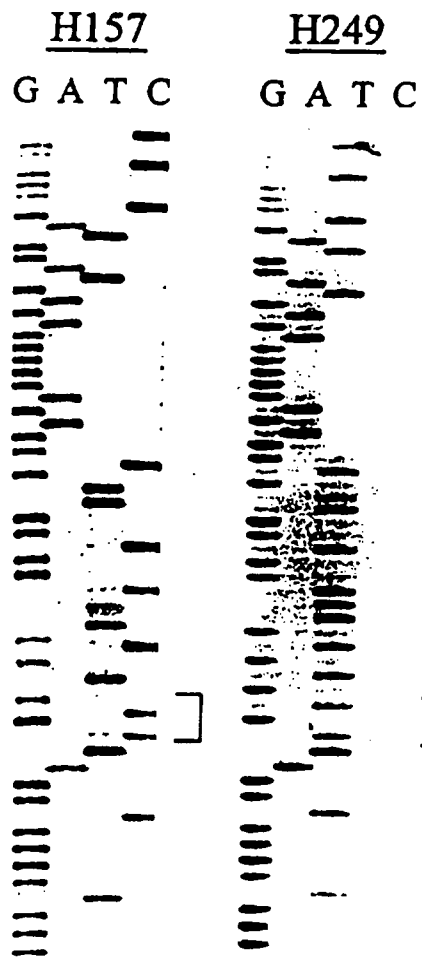
Figure 1

Figure 2

Figure 3

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/68; C07H 21/04; C12P 19/34
US CL :435/6, 91.2; 536/24.33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CA, DERWENT
search terms: methylation, PCR, primers, bisulfite, TIMP-2, RNA expression

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y ---- A	FROMMER et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc. Natl. Acad. Sci. USA. March 1992, Vol. 89, pages 1827-1831, especially pages 1828-1829.	16-19 and 22-24 ---------------- 1-15, 20, 21, and 25- 28
A	STETLER-STEVENSON et al. Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. J. Biol. Chem. 15 August 1990, Vol. 265, No. 23, pages 13933-13938.	29-36
A	US 5,324,634 A (ZUCKER) 28 June 1994	29-36
A,P	US 5,595,885 A (STETLER-STEVENSON et al.) 21 January 1997.	29-36

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 AUGUST 1997	2 9 AUG 1997

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer KENNETH R. HORLICK
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